Comparison of Human Cytomegalovirus Entry Mechanisms into Porcine and Human Endothelial Cells

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"In the end, everything will be ok.
If it's not ok, it's not yet the end"
Fernando Sabino

Table of contents

1 Summary

Over the past decades, allotransplantation has evolved from an experimental procedure to a very successful, often life-saving therapy and has emerged as the best therapy to treat patients with end-stage organ failure. The success of this surgical intervention has led to an increasing demand limited by the availability of organs. To overcome the acute shortage of human organs, xenotransplantation has become a focus of interest and a very important area of transplantation research. Miniature pigs have evolved as the most suitable source of organs for several reasons including: housing in a pathogen-free environment, the capacity to breed and have a big number of offspring and last but not least some similarities shared with human organs regarding size and physiology. Currently, clinical pig-to-human trials involving porcine islet cells are performed with encouraging results since patients have seen their daily dose of insulin reduced and one of the patients is said to be insulin-free. However, because of a high immunosuppression treatment to prevent graft rejection, the transplanted organ will inevitably be in contact with pathogens. Concern has mainly been focused on donor-derived xenogeneic infection, neglecting host-derived infection with their potentially severe consequences for the xenograft including rejection and loss of function. Primary infection and reactivation of human cytomegalovirus (HCMV) is the most important viral complication post-transplantation and has been associated with allograft rejection. Our laboratory has previously demonstrated that HCMV cross-species infection of porcine cells induces atypical cytopathic effect and induces apoptosis, which may eventually result in porcine xenograft rejection. The goal of my thesis was to investigate potential HCMV entry pathways in porcine endothelial cells, in order to develop strategies for prevention and to compare it with human endothelial cells, yielding potentially new insights on the mechanisms of HCMV entry.

My findings demonstrate that similarly to entry into human cells, entry of both the endotheliotropic (TB40/E) and the fibrotropic (TB40/F) (except for microvascular porcine endothelial cells) strains into porcine endothelial cells (pEC) requires the expression of the β1 integrin subunit. In contrast, the expression of platelet-derived growth factor receptor alpha (PDGFRα) is required for entry of both strains in pEC only. I demonstrate that internalization of TB40/E viral particles by human

endothelial cells and pEC occurs with a similar mechanism, i.e. a dynamin-2-, lipid rafts-, actin- and pH-dependent mechanism, whereas internalization of TB40/F viral particles by pEC occurs mainly by a dynamin-2-dependent, clathrin-, lipid rafts-independent mechanism and in a pH dispensable manner. Besides, when actin polymerisation is prevented, TB40/F enters pEC in an actin-independent fashion, suggesting an entry by fusion or the involvement of another component of the cellular cytoskeleton. I demonstrate for the first time that after internalization, TB40/E viral particles translocate to the nucleus of hEC through the classical endolysosomal pathway going from early endosomes to late endosome / lysosomes. Similar results were observed for pEC. In contrast, in TB40/F-infected hEC, viral particles located only in early endosome, suggesting a sequestration of virions in this compartment, whereas in pEC, viral particles were able to transfer from early endosomes to late endosomes / lysosomes. Finally, I demonstrate that nuclear translocation differs between hEC and pEC and viral strains, as observed by a nuclear accumulation of the tegument protein pp65 only in TB40/F-infected pEC. This differential nuclear accumulation is correlated with a higher accumulation of pp65 in TB40/F-infected EC compared to TB40/E-infected EC, which results in an earlier initiation of replication of TB40/F compared to TB40/E in pEC.

In conclusion, my findings suggest that HCMV uses distinct entry pathways that are dependent on both the strain and the origin of the target cells, underlying the capacity of HCMV to adapt to its environment, and its ability, following entry, to induce a successful infection of pEC challenges the traditional paradigm of species specificity. Besides its fundamental research significance, investigation of HCMV cross species infection has an important implication when applied to xenotransplantation. Determining the early events of infection will help in developing strategies to prevent HCMV infection by blocking entry into porcine cells or by rendering swine resistant to HCMV infection, which is essential for xenotransplantation to become a clinical reality.

2 Zusammenfassung

Die Allotransplantation hat sich von einem experimentellen Verfahren zu einer sehr erfolgreichen, häufig lebensrettenden Behandlung von Patienten mit terminalem Organversagen entwickelt. Dieser Erfolg hat zu einer zunehmenden Verknappung von Organen geführt. Als Alternative ist die Xenotransplantation in den Blickpunkt des Interesses gerückt. Miniaturschweine werden als die aktuell besten Spender angesehen, weil diese verschiedene Voraussetzungen erfüllen: Pathogen-freies Züchten und Halten der Tiere, eine relative kurze Tragzeit und Nachkommen in genügender Anzahl. Weitere Vorteile sind eine ähnliche Physiologie und Grösse der Organe. Aktuell gibt es weltweit klinische Versuche mit Transplantation porziner Inselzellen in Menschen zur Behandlung der Diabetes; diese Versuche sind vielversprechend. Für die Organtransplantation ist davon auszugehen, dass für die Verhinderung der Abstossung eine erhebliche immunsuppressive Therapie notwendig sein wird. Dabei steht das transplantierte Organ in engem Kontakt mit potentiellen Pathogenen. Bisher waren die Bedenken auf eine Uebertragung von vom Spender ausgehenden Infektionen ausgerichtet, sog. xenogenen Infektionen. Dabei wurde zu wenig berücksichtigt, dass humane Pathogene das transplantierte Organ ebenfalls befallen und schädigen können, mit dem Resultat eines Organversagens. Die Infektion mit Zytomegalievirus spielt in diesem Zusammenhang eine wichtige Rolle, ist sie doch die häufigste virale Komplikation nach Allotransplantation. Unser Labor konnte in früheren Studien darlegen, dass menschliche Zytomegalieviren porzine Zellen infizieren können. Diese Infektion führte zu Veränderungen des Phänotyps mit einer alterierten Expression verschiedener Marker und zu zytopathischen Effekten und Induktion der Apoptose. Diese Veränderungen haben das Potential, in einem transplantierten Organ eine Abstossung oder eine Funktionsverlust zu induzieren.

Ziel dieser Arbeit war es, die Mechanismen des Eindringens von Zytomegalieviren in porzine Endothelzellen zu studieren. Ein besseres Verständnis würde es erlauben, Strategien zur Verhinderung einer solchen Infektion zu entwickeln.

Meine Resultate demonstrieren, dass wie im menschlichen Modell mit humanen Endothelzellen sowohl endotheliotrope (TB40/E) als auch fibrotrope (TB40/F) Stämme für eine erfolgreiche Infektion den Korezeptor β1 Integrin

benötigen. Auf der anderen Seite ist PDGFRα (platelet-derived growth factor receptor alpha) notwendig in porzinen, nicht aber in humanen Zellen. Internalisierung von TB40/E erfolgt sowohl in menschlichen als auch porzinen Zellen durch ähnliche Mechanismen, so ist dieser Prozess Dynamin-2-, lipid rafts (Cholesterin-reiche Mikrodomänen in Zellmembranen)-, Aktin- und pH-abhängig. Beim fibrotropen Stamm (TB40/F) erfolgt dieser Prozess in porzinen Zellen mittels eines Dynamin-2-abhängigen, aber Clathrin-, lipid raft-unabhängigen Mechanismus, auch schient der pH keine Rolle zu spielen. Aktin ist für das Eindringen von TB40/F in porzine Zellen nicht notwendig. Ich konnte ebenfalls zum ersten Mal im menschlichen Modell zeigen, das TB40/E nach Eindringen den klassischen endolysosomalen Weg zum Zellkern nimmt, und dass dies auch für porzine Zellen gilt. Ein anderes Verhalten wies der fibrotrope Stamm TB40/F auf: In menschlichen Ednothelzellen konnte dieser nur in einem frühen Lysosm nachgewiesen werden, während in porzinen Zellen der ganze Transportweg nachverfolgt werden konnte. Mittels des Nachweises der Akkumulation eines viralen Tegumentproteins, pp65, konnten wir nachweisen, dass der Transfer zum Nukleus differiert zwischen humanen und porzinen Endothelzellen, was zu einer früheren und gesteigerten Replikation von TB40/F im Vergleich zu TB40/E in porzinen Endothelzellen führt.

Zusammenfassend zeigen meine Resultate auf, dass Zytomegalieviren eine Vielzahl verschiedener Möglichkeiten aufweisen, Zellen zu infizieren. Dabei sind die Unterschiede nicht primär zwischen Zellen verschiedener Arten sichtbar, sondern vielmehr abhängig von der jeweiligen anatomischen Abstammung der Zelle und vom Virusstamm. Diese Erkenntnisse zeigen die enorme Adaptationsfähigkeit von Zytomegalieviren auf, sich in verschiedenen Zellen zu replizieren und infektionsfähige Nachkommen zu generieren. Damit ist auch das lang-gehegte Paradigma der Spezies-Spezifität in Frage gestellt. Diese Resultate haben neben der biologischen Bedeutung auch die praktische Konsequenz, dass nach Transplantation eines porzinen Organs im Menschen mit einer Infektion durch humane Zytomegalieviren gerechnet werden muss. Der Verhinderung einer solchen Infektion kommt dabei zentrale Bedeutung zu, bevor Xenotransplantation eine klinische Realität wird.

Introduction, part 1:

Human Cytomegalovirus

3 Introduction

3.1 Discovery of human cytomegalovirus (HCMV)

In 1881, German scientists described for the first time intranuclear inclusions in sections of the kidney of a stillborn infant with congenital syphilis and in the parotid gland of children (232). They attributed this discovery to protozoa after seeing a report from Jesionek and Kiolemenoglou describing similar cells as "protozoan like" cells in the lungs, kidneys and liver of a luetic foetus (136). In the 1920's, Von Glahn and Pappenheimer suggested a viral etiology by linking these cellular abnormalities with intranuclear inclusion containing cells in lesions in man infected by herpes zoster and herpes genitalis (303). The isolation of the viral agent became possible only after human cells were routinely grown in cell culture. Three independent groups isolated the viral agent in the 1950's. In 1955, Margaret Smith isolated a virus from the salivary gland of a dead patient that grew only in human cells but not in murine cells. Her paper was rejected because she was working in parallel with a murine salivary gland virus and a possible contamination was suspected. One year later her discovery was published after she re-isolated the same agent from another kidney from a patient (268). That same year, Rowe isolated the same agent from adenoid tissue of a child, and this strain was then referred to as AD169 (234). Finally, Weller isolated the virus from a patient suspected to have toxoplasmosis (58) and in 1960 he named it cytomegalovirus.

Figure 1: Representation of cells with intranuclear inclusions suspected to be parasites as decribed by Jesionek and Kiolemenoglou (left) (136) or CMV-infected cytomegalic cells (right).

3.2 Epidemiology of HCMV infection

3.2.1 Clinical relevance

HCMV is observed in all geographic locations and socio-economic groups and infects 50-90% of the adult population. Seroprevalence increases with age (7) and is higher in developing countries and in communities with a low socio-economic status. Like all herpesviruses, HCMV establishes a life-long latent infection after primary infection. In immunocompetent hosts, primary infection is asymptomatic or induces a mononucleosis-like syndrome. However, in rare cases, HCMV infection can lead to severe illness involving different organs, resulting for instance in hepatitis, neuropathies, encephalitis, myocarditis and ocular disease (75, 159). By contrast, in immunocompromised individuals, including neonates, AIDS patients, and transplant recipients, HCMV infection often causes life-threatening diseases, including pneumonia, gastrointestinal disease, hepatitis, retinitis, and encephalitis, resulting in significant morbidity and increased mortality (49, 217, 228, 280).

In human immunodeficiency virus (HIV) positive patients, HCMV is an important opportunistic pathogen. In advanced HIV diseases with low CD4 T helper cell numbers, CMV disease can reactivate, with retinitis being the most common clinical manifestation. Retinitis reduces the visual acuity and the damages are irreversible (250). If not treated, retinitis expands to both eyes and later HCMV spreads to other organs with the development of encephalitis. Since the introduction of antiretroviral therapy (ART), the incidence of HCMV retinitis has dramatically declined (67), and today, most cases of HCMV retinitis occur in patients where HIV is diagnosed late in the course of disease or who are unable to control the infection (no access to ART, etc.)

Congenital HCMV infection remains an important public health problem causing significant morbidity and mortality. Primo-infection or infection with another strain occurring early in gestation can lead to spontaneous abortion (110). Although most neonates infected *in utero* do not show any signs of infection (204), complications may occur in the first year including hearing loss, mental retardation and visual abnormality (37, 157). When transmission occurs later in pregnancy, it is associated with premature delivery and intrauterine growth retardation (133).

HCMV infection is also involved in other diseases such as atherosclerosis (thickening of artery wall as a result of the accumulation of fatty materials such as cholesterol and triglyceride). HCMV infection stimulates cellular proliferation and increases angiogenesis (16). Several contradictory studies about the detection of HCMV in normal and atherosclerotic samples leave some doubts on the involvement of HCMV infection in atherosclerosis (318, 320). However, more recently, a survey demonstrated that CMV seropositivity and inflammation are associated with higher risk of cardiovascular disease-related death (260).

Finally, in transplant recipients, HCMV is the most important viral infectious complication and is associated with allograft rejection. HCMV infection in solid organ transplant recipients often manifests by HCMV syndrome (fever, anorexia and malaise), and is often linked with dysfunction of the organ transplanted: hepatitis in liver recipients, pancreatitis after pancreas transplantation, myocarditis in heart recipients and pneumonitis in lung or lung and heart recipients (170). Indirect effects are also observed such as a decreased efficiency of the immune system leading to the development of opportunistic infections, and allograft rejection (170).

3.2.2 Transmission of HCMV

HCMV is an enveloped virus, which renders it very fragile and unable to persist in an external environment. As a consequence, transmission occurs by close contact with seropositive persons (sick or asymptomatic) excreting viral particles in oropharyngeal secretions, urine, semen, vaginal and cervical secretions, milk, saliva and tears (7, 119, 162, 231). Several modes of transmission have been described.

HCMV transmission from mother to infant can occur at two different stages: during pregnancy *in utero* (congenital infection) or during or after delivery (perinatal / postnatal infection). Congenital CMV infection results from transplacental transmission of the virus during maternal viremia (8). Perinatal / postnatal HCMV infection from the mother to the child may occur through the genital tract during delivery or through breast milk, the later mode of transmission being the most spread in the world (73, 231, 275, 276).

Children also acquire HCMV from other children or indirectly through environmental contamination (toys for instance). Group day care appears likely to result in early acquisition of HCMV. Pass et al. demonstrated that children in day care shed virus in saliva and urine, exposing virus to other children, nursery workers and

mothers (210, 211). The analysis of HCMV DNA isolated from infected children attending the same day care centre supports child to child transmission. Adler examined the HCMV DNA pattern following restriction endonuclease digestion of 16 children from the same day care centre and he found out that two groups of children were excreting the identical viral strains (3). Hugo et al. showed that HCMV survives 30 minutes on toys and the analysis of children's oral behavior showed that every 2 to 3 minutes they put toys and hands in their mouth (129), favoring HCMV environmental transmission.

In adults, transmission mostly occurs through sexual activity because of the presence of the virus in genital tract. HCMV antibody prevalence in teenagers doubles during the first year of sexual activity (311). Seropositivity is related to the number of sexual partners and the age of onset of sexual activity (50, 214, 256, 270).

Finally, the last mode of transmission in industrialized countries is the iatrogenic infection, which includes blood transfusion or organ transplantation from a seropositive donor. In 1983, Adler reported that i) seropositive blood donors are the source of HCMV infection for seronegative patients and that ii) the number of donors and the volume of blood transfused to recipients impact the incidence of HCMV infection (4). Gilbert et al. demonstrated that the leukocytes are the components responsible for infection; therefore filtration of blood through a leucocyte filter can prevent HCMV infection (97).

3.2.3 Treatment of HCMV infection

The treatments described in this section are usually given to immunocompromised patients such as transplant recipients and HIV patients, as in the immunocompetent patient, HCMV is usually not treated.

There are currently three clinical strategies for the management and prevention of CMV disease in immunocompromised individuals: i) the prophylactic therapy, which consists in treating all patients irrespective of the risk of CMV disease before viral replication is detected, ii) the pre-emptive therapy, which is given to patients with a high risk of future disease where early viral replication is occurring and iii) treatment, which consists in treating patients showing evident clinical symptoms (223).

Table 1: Synopsis of the timing of the administration of the different strategies. Adapted from Griffiths et al, JAC, 2003 (109).

Term used	When drug given	Risk of disease	Acceptable toxicity
Treatment	Once disease apparent	Established	High
Pre-emptive therapy	After systemic detection	High	Medium
Prophylactic therapy	Before active infection	Low	Low

To date, four antiviral agents have been licensed: ganciclovir (GCV), its prodrug valganciclovir (VGCV), foscarnet (257) and cidofovir (CDV). Intravenous GCV and oral VGCV are used as a first-line treatment and FOS and CDV are used as alternatives to the first two drugs. These two second line drugs have a potential for significant toxicity (renal or hematologic toxicity), therefore only patients with established CMV disease are treated. All four drugs target the viral DNA polymerase (47, 66, 269) and prolonged antiviral treatment has led to the development of HCMV cross-resistant strains (46, 116, 267, 282); therefore development of new drugs is needed. Some candidates have been studied with diverging outcomes. Maribavir had promising results with several Phase I and II trials which revealed good bioavailability and tolerability (18, 79, 296), however a recent Phase III trial showed some limitations (180) and the occurrence of a resistant strain was reported (279). The latest drug, in clinical development, Letermovir (known as AIC246), interacts with the UL56 subunit involved in viral DNA processing and packaging. As for now, no resistance has been reported (101, 167).

3.3 General characteristics

HCMV, alternatively known as Human Herpesvirus 5 (HHV-5), belongs to the *Herpesviridae* family, which contains 8 viruses known to cause disease in humans and which are classified in 3 subfamilies: *alpha-*, *beta-* and *gammaherpesvirinae*. HCMV belongs to the *Betaherpesvirinae* subfamily, which is characterized by a long replication cycle in cell culture and traditionally, a strict species specificity. HCMV has a divergent cell tropism, however, in human hosts, although HCMV infects nearly all differentiated cell types, including fibroblasts, hematopoietic, endothelial and epithelial cells (reviewed in (263)), a few cell types do not support productive infection counting lymphocytes and polymorphonuclear leukocytes (106, 264). Tropism is not determined by the expression of entry receptors on the cell surface,

rather, non-permissive cells allow viral binding and internalization but block viral replication at a post-penetration step (263, 265).

Table 2: Viruses from the *Herpesviridae* family causing disease in humans (213). [a] Values obtained in different laboratories.

Type	Synonym	Subfamily	Genome size (kbp)
HHV-1	Herpes Simplex 1	α	152
HHV-2	Herpes Simplex 2	α	155
HHV-3	Varicella zoster virus	α	125
HHV-4	Epstein Barr virus	γ	172
HHV-5	HCMV	β	235
HHV-6A and 6B	Roseolovirus, Herpes lymphotropic virus	β	159-170[a]
HHV-7		β	145
HHV-8	Kaposi's sarcoma-associated herpesvirus	γ	170-210[a]

3.3.1 Virion structure

HCMV virion structure is typical of other herpesviruses although it is larger and has a more irregular envelope (168, 192). HCMV genome is composed of a double stranded DNA molecule of ~235 kbp contained in an icosahedral capsid of 125 nm enclosed in a proteinaceous layer called tegument (or matrix). The tegument is surrounded by a host cell derived lipid bilayer envelope carrying a large number of virus-encoded glycoproteins, and all together, the components generate viral particles ranging from 200 to 300 nm in diameter (130, 190).

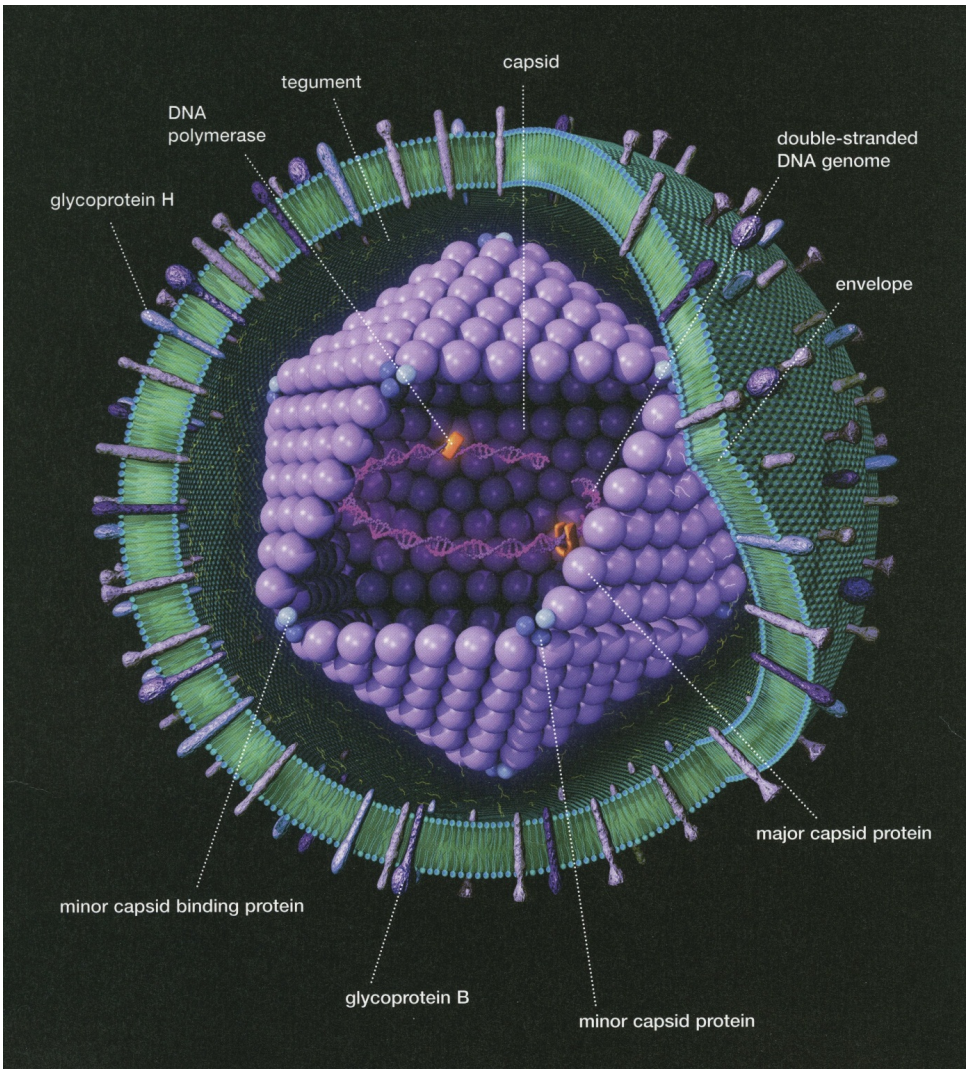

Figure 2: Structure of a HCMV viral particle (Image reproduced from Human Cytomegalovirus, Human virus guides 3; Edited by Vincent C Emery, International Medical Press, 2007)

3.3.1.1 The genome

The HCMV genome is the largest of all herpesviruses. It is composed of two unique regions of DNA: a unique long sequence (UL) and a unique short sequence (US) flanked by internal and terminal repeated sequences. The orientation of UL and US, due to recombination between flanking inverted repeated sequences, promotes the production of 4 distinct isomers (261). In total the genomes encodes for 200-220 genes, depending on the viral strains (clinical versus laboratory strains). This discrepancy is explained by the extensive passages in fibroblasts of the laboratory strains, which led to a large deletion (the sequence comprised between UL128 and UL131) and the accumulation of punctual deletions and mutations in laboratory strains (32, 71, 198).

3.3.1.2 The nucleocapsid

The nucleocapsid is organized on a T=16 icosahedral lattice which is composed of 150 hexons (capsid faces) and 12 pentons (capsid vertices). The HCMV nucleocapsid is composed of 4 structural proteins (192):

- The major capsid protein (MCP), encoded by the gene UL86, is the main component of the nucleocapsid (36) and forms the hexons and pentons of the icosahedral capsid,
- The minor capsid protein (mCP), encoded by the gene UL85, forms the trimers (together with the minor capsid binding protein) that connect hexons and pentons together,
- The minor capsid protein-binding protein (mC-BP), encoded by the gene UL46, interacts with mCP (96) and forms trimers,
- The smallest capsid protein (SCP), encoded by a sequence between the genes UL48 and UL49 (referred to as UL48/49), decorates the outside of the nucleocapsid and binds MCP on the tip of hexon subunits (158, 323). SCP is also essential for HCMV infection *in vivo* (20).

Besides these structural proteins, nucleocapsid contains several internal, closely related proteins, which catalyse the assembly process: the assembly protein (AP) and proteinase precursors (also known as assemblin) both encoded by different regions of the gene UL80. The AP precursor (pAP), which supplies the nuclear localization signal (NLS), interacts with MCP in the cytoplasm, with enables the complex MCP/pAP to translocate to the nucleus (220, 317). The assemblin, which have counterparts among all herpesviruses, is important for assembly and maturation of the nucleocapsid and is essential for the production of infectious viral particles (72, 95, 310).

3.3.1.3 The tegument

Approximately 20 to 25 known virion-associated tegument proteins have been identified; most of these proteins are phosphorylated (192) and highly immunogenic (24). Amongst them, the most abundant tegument proteins are pp65 (encoded by the gene UL83), pp150 (encoded by the gene UL32) and pp71 (coded by the gene UL82), which represent 15%, 9% and 9% of the virion mass, respectively (302). The tegument protein pp65 contains two NLS, which allow it to translocate to the nucleus minutes after infection (91). The expression of pp65 occurs early during infection.

Once expressed, pp65 accumulates in the nucleus and finally translocates to the cytoplasm at late times post-infection to be incorporated in the virions (121). One function of pp65 is its capacity of both autophosphorylation and phosphorylation of immediate early (IE) viral proteins by its serine/threonine kinase activity, preventing the cleavage of IE and subsequent presentation through the major histocompatibility complex class 1 molecules (MHC-I) (98). The transactivator tegument protein pp71 activates the IE gene expression (273) and is necessary for efficient viral replication (reviewed in (141)). pp150 plays a role in the assembly and egress of virus particles since it is necessary to incorporate nucleocapsids into virus particles (302).

Besides proteins, the tegument contains as well cellular and viral RNAs that are incorporated non-specifically during virion assembly (22, 107).

3.3.1.4 The envelope

HCMV particles have a lipid bilayer envelope derived from the endoplasmic reticulum (ER) or ER Golgi intermediate complex (ERGIC) (126). The envelope contains three major glycoprotein complexes:

- The glycoprotein B (gB), encoded by the gene gpUL55. gB is expressed as a disulphide-linked homodimer and the resulting complex is named glycoprotein complex I (gcI). This complex has several functions including: i) binding to heparin sulphate proteoglycans (HSPG) and receptors expressed on the cell surface of target cells (21, 53, 81, 190, 307), and ii) cell-to-cell spread infection (203); although a recent study suggests that the main role of gB is to induce fusion rather than binding receptors (315). gB is a major target of neutralizing antibodies, making it the favourite candidate for the elaboration of vaccines (191, 192, 274).
- The disulphide-linked dimer gM:gN (also referred to as gcII), the most abundant protein component of the envelope, is encoded by gpUL100 and gpUL73, respectively. This complex has also several functions including: i) binding to HSPG on the target cells (142), ii) implication in assembly and replication (gM) (154), and iii) protection of the virus from neutralizing antibodies (gN) (153).
- The heterodimer complex gH:gL (also referred to as gcIII) encoded by gpUL75 and gpUL115, respectively, is important for fusion (51, 52,

153). gH:gL can interact with two distinct component, gO or the complex UL128-131 which is responsible for HCMV tropism. In endotheliotropic HCMV strains, gH:gL interacts with UL128-131, allowing viral particles to enter and replicate into epithelial and endothelial cells (amongst others) (2, 93, 115, 238, 239, 305). In contrast, fibrotropic HCMV strains acquired mutations in the UL128-131 genes and cannot assemble gH/gL/UL128-131 and, therefore, cannot infect endothelial and epithelial cells, leukocytes, and monocytes (32, 71, 115). gO is important for HCMV entry into fibroblasts (300) and is involved in the incorporation of the complex gH :gL in viral particles and the virus release (137, 237, 314).

3.3.2 HCMV replication cycle

As for other herpesviruses, genes are transcribed by the cellular RNA polymerase II and gene expression occurs via a temporal cascade which counts an immediate-early (IE), an early (E) and a late (L) phase. The expression of IE proteins follows virus entry and does not require the expression of any other viral proteins. IE1 and IE2 encoded by the genes UL123 and UL122, respectively, are the main IE proteins and are essential for the regulation of the expression of E and L proteins.

The expression of E proteins occurs 4-12 hours post-infection. During this E phase, non-structural proteins important for viral replication, packaging and maturation of viral particles are produced. These E proteins include amongst others: the viral DNA polymerase (encoded by UL54), a viral DNA polymerase processivity factor (encoded by UL44) and the protein kinase (encoded by UL97), the latter controls the phosphorylation of the antiviral drug GCV (172, 283).

Finally, during the L phase, the viral genome is replicated and viral structural proteins are expressed. The essential functions of these L proteins are nucleocapsid maturation, DNA encapsidation, virion maturation and egress of viral particles from the cell.

3.4 HCMV tropism and virus entry

Herein, I will define “entry” as all the processes from the initial binding of viral particles to the initiation of replication with the expression of IE proteins.

3.4.1 HCMV factors associated with viral tropism

Tropism differs between clinical and laboratory strains. As written in chapter 3.3.1.4, the difference observed in the tropism is based on the loss during several passages on fibroblasts of the loci UL128, UL130 and UL131A in laboratory strains (135), which are major viral determinants of HCMV tropism. The presence of the complex UL128-131 on the viral envelope of endotheliotropic strains, and its association with the complex gH:gL (gH:gL:UL128-131) allows HCMV to enter epithelial and endothelial cells (115, 236). Wang and Shenk demonstrated that an intact UL128-131 locus in AD169 (after repairment of the gene UL131) is important for infection of both epithelial and endothelial cells (304), and Ryckman showed that the complex gH:gL:UL128-131 plays an important role in endocytosis and pH-dependent entry into these cells (238). In contrast, in fibrotropic strains, the absence of this complex and the presence of another complex (gH:gL:gO) allows HCMV entry into fibroblasts (300). It has been recently demonstrated that the cell type in which viral strains are produced plays a major role in the determination of the tropism of progenies. When an endotheliotropic strain is grown in fibroblasts, progenies infect both fibroblasts and endothelial cells in a cell-free virus manner, whereas when the same strain is grown in endothelial cells, progenies infect only fibroblasts in a cell-to-cell manner. This difference was explained by the amount of UL128-131 incorporated in virions where fibroblasts-derived progenies displayed higher amounts of this complex compared to endothelial cells-derived progenies (253).

3.4.2 HCMV entry mechanisms

In human hosts, HCMV infects nearly all cell types, including fibroblasts, hematopoietic, endothelial and epithelial cells (reviewed in (263)). This extensive host cell range correlates with the potential for clinical manifestation of HCMV disease in almost every organ. This broad cellular tropism suggests that, to enter human cells, HCMV might use receptor(s) common to most cells (52). The entry of HCMV into human cells is a complex process requiring sequential interaction between multiple cellular and viral components.

On many HCMV-permissive cell types, HCMV glycoproteins including gB homodimers and gM:gN heterodimers, first transiently attach to HSPG (i.e. the tethering step) (55) before stable attachment to and entry through transmembrane

protein receptors (51). HCMV enters cells via two distinct mechanisms depending on the cell type. In human fibroblasts it occurs by a pH-insensitive membrane fusion (54) whereas in human epithelial and endothelial cells, the virus enters via endocytosis in a pH-dependent manner (19, 238). Both fusion and endocytosis display common steps in HCMV entry involving cellular receptors. Numerous cellular receptors have been described, although, none appears to be required in all cell types. These receptors include epidermal growth factor receptor (EGFR) (34, 131, 308), platelet-derived growth factor receptor alpha (PDGFRα) (272, 301) and various integrin family heterodimers: α2β1, α6β1, αvβ3 integrin (81, 305).

The cellular cytoskeleton plays an important role for entry and nuclear translocation of many viruses. It is composed of three different components, namely microtubules, actin microfilaments and intermediate filaments (IF). Entry and trafficking of herpesviruses was shown to be dependent on both the actin and the microtubule cytoskeleton, which cooperate in order to deliver the viral genome to the nucleus on infected cells (173). In the case of HCMV, internalization of the viral particle requires an intact actin cytoskeleton (19). Following viral internalization and release of the nucleocapsids into the cytoplasm, the nucleocapsids associate with the microtubule cytoskeleton and are transported towards the microtubule-organizing centre (MTOC) located near the nucleus (207). However, one must be cautious in the interpretation of such results since inhibitors of microtubules and microfilament affect IF organization (224). Currently little is known about the involvement of IF in virus entry, however, in 2009, Miller et al. reported that an intact vimentin cytoskeleton, an IF protein, is required for HCMV entry into fibroblasts (186).

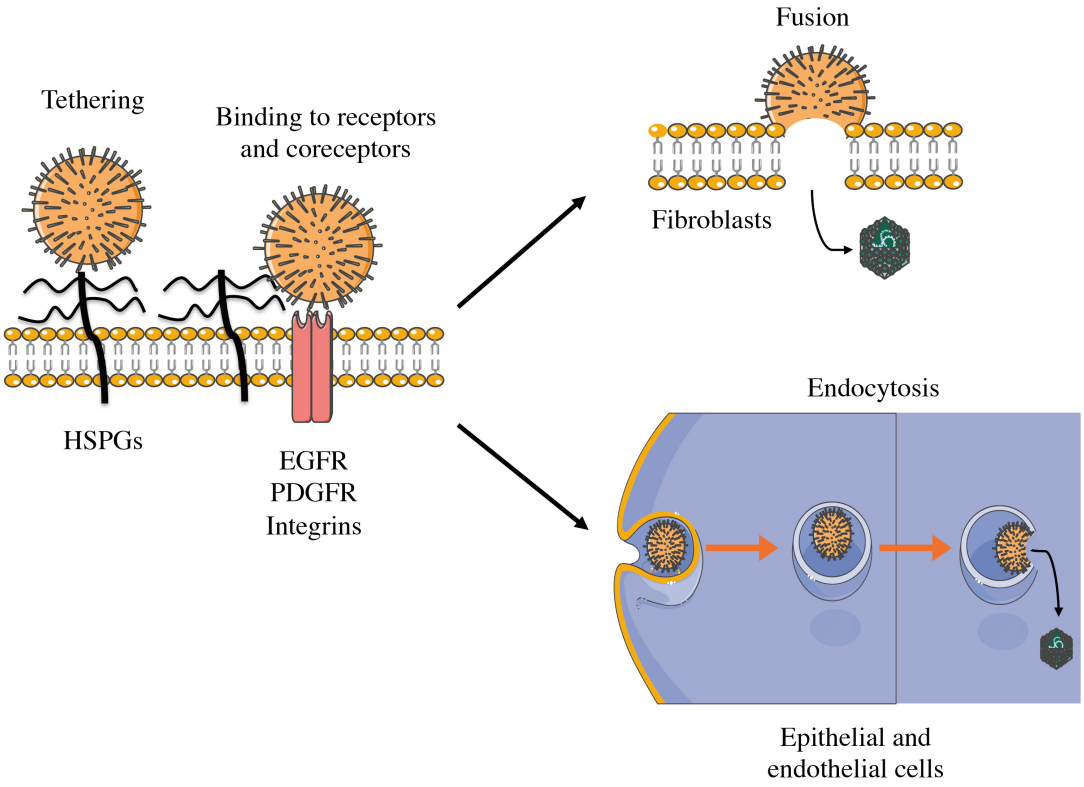

Figure 3: Cartoon illustrating the entry steps of HCMV in human cells (illustration created thanks to Servier Medical Art, http://smart.servier.fr/servier-medical-art).

3.5 HCMV cross-species infection

Cytomegaloviruses are found in many mammalian species and have coevolved with their respective hosts, which has led to the traditional paradigm that CMVs are generally considered to be highly species-specific since their first isolation in cell culture in 1955. Several groups demonstrated that replication of CMVs can occur in cells of their own or a closely related species. In 1988, Lafemina et al. demonstrated that CMVs (simian CMV, mouse CMV and HCMV) enter cells from the other species and start replicating, which was observed by the production of IE proteins (156). Later, in 1992, Perot et al. demonstrated that chimpanzee cells are permissive to HCMV (216). Similar results were observed for mouse CMV (MCMV) and rat CMV (RCMV) (25, 266) where it was shown that MCMV replicates in rat cells but rat CMV fails to replicate in murine fibroblasts.

Although cells of more distant species are usually non-permissive, it has also been reported that CMVs can enter these cells and express a subset of viral genes, which is followed by either a reduced release of infectious viral particles when compared to human fibroblasts (216), or an abortive infection (83, 146, 156). These

findings suggest that the restriction of CMV replication in non-permissive cells is due to a post-penetration block rather than a failure to enter cells. More recently, the molecular mechanism underlying CMVs specificity was investigated and several mechanisms have been described. Jurak et al. have demonstrated that MCMV infection of human cells induces apoptosis, and that inhibition of apoptosis enables as well rat cytomegalovirus to cross the species barrier and replicate into human cells, indicating that this mechanism is not specific to one type of CMV (140). More recently, the group of Tang assessed the involvement of the nuclear structure called ND10 (nuclear domain 10), also referred to as promyelocytic leukaemia nuclear bodies (PML bodies), in limiting cross-species infection. Several herpesviruses, including CMVs are capable of disrupting this domain (6, 132), whose components have been shown to have negative impacts on herpesviruses (125, 225, 240, 290), and various viral proteins have been identified as being related to ND10 and ND10 proteins (reviewed in (241)). They demonstrated that during a cross-species infection, ND10 are dispersed by CMVs and that ND10 components are involved in blocking viral gene expression in such infections (57).

Introduction, part 2:

Xenotransplantation

3.6 History of transplantation

3.6.1 Allotransplantation

Allotransplantation consists in transferring cells, tissues or organs between two genetically non-identical individuals of the same species. Over the past decades, allotransplantation has evolved from a "clinical experiment" to a very successful, often life-saving therapy and has emerged as the best therapy to treat patients with end-stage organ failure.

The first notions of tissue transplantation were already described several millennia ago in ancient literature. For instance, the Sushruta Samhita, a Sanskrit text about surgery, described how Sushruta, one of the earliest surgeons known in history, practiced, in 600 BC, nasal reconstruction. In ancient times, nasal amputation was a common punishment for criminals and Sushruta would reconstitute patient's nose with their own skin grafts cut from their cheek. According to their hagiography, in the 3rd century AD, two Saints, St. Cosmas and St. Damian performed a leg transplantation to replace a cancerous leg with a leg from a dead black man (56). By the early 20th century, the French surgeon Alexis Carrel, pioneer of the vascular suturing technique and creator of the "perfusion pump" (a device allowing living organs to exist outside of the body during surgery) successfully reimplanted a kidney in the neck of the same dog. A few years later he reiterated the surgery between dogs and by his observations he concluded that the host response to the foreign graft (i.e. the immune system) was responsible for the allograft rejection (29-31). He was awarded in 1912 with the Nobel Prize in Physiology or Medicine for his work on vascular suture and the transplantation of blood vessels and organs. After the World War I, several kidney transplantations were performed but all of them failed due to rejection. Starting from 1950s, successful kidney transplantations were documented. The surgical team represented by Dr. Joseph Murray and Dr. John Merrill successfully transplanted on two occasions a kidney between two identical twins. First in 1954 between twin brothers whose recipient survived 8 years with a fully functional kidney (183); and second in 1956 between twin sisters whose recipient survived 55 years making her the longest surviving transplant recipient (199). It was becoming evident that the suppression of the recipient's immune system was the key to allograft rejection. In the late 1950's, one approach to suppress the recipient's

immune system was to irradiate it. Patients were treated with sub-lethal radiations; however most of the patients' cause of death was linked to the effects of radiation rather than rejection. The discovery and development of immunosuppressive agents 10 years later opened an alternative possible pathway. In the 1960's, Dr. Thomas Starzl demonstrated that a "cocktail" of immunosuppressive drugs was able to reverse allograft rejection and induce host tolerance towards the graft (278). This discovery opened the door to the transplantation of organs other than kidneys such as lungs, pancreas, heart and liver.

The success of allotransplantation has led to a shortage in the supply of organs required to meet the increasing demand. In 2012, 1165 persons were in need of a transplant in Switzerland and nearly 10% died awaiting for an organ (Swisstransplant). The solution to this shortage is to find another kind of donor whose transplantation would be compatible with human. Xenotransplantation, defined as the transplantation of living cells, tissues or organs from one species to another, might be one possibility. Nowadays it has become a focus of interest and a very important area of transplantation research.

3.6.2 Xenotransplantation

The idea of a part man – part animal chimera has always fascinated men since several millennia. In mythology, gods of the Ancient Egypt were often represented with the body of a man and the head of an animal. Ra, god of Sun had the head of a falcon; Anubis, god of mummification and afterlife had the head of a jackal and Sobek, god of the Nile was seen as a crocodile-headed man. In Ancient Greece, several mythical monsters were also part human – part animal. Amongst others, centaurs were horses with the trunk and the head of a man, the Minotaur was a man with the head of a bull and the harpies were birds with the head of a woman. Cross-species transplantation (or xenotransplantation) recipients can be considered as today's chimera. The first xenotransplantation attempts were made without any knowledge of the species barrier, resulting in major failures. At first, cells and tissues comprised the majority of the transplants and later, with the mastering of the technique of vascularization of the graft and the development of immunosuppression, xenotransplantation of organs followed.

In 1501, Mohammad Baha' al-Dawla, an Iranian surgeon, performed the first bone tissue xenotransplantation by transplanting a patient suffering from an

osteomyelitis of the skull with a piece of a dog's bone. In 1667, the first successful xenotransfusion was documented; a man suffering from severe fever was cured after being transfused with blood from a lamb (80). In the end of the 19th century, testicle xenotransplantation from ape to human was used as a "human revitalization transplantation" (64). In 1889, Charles-Edouard Brown-Séquard was a physician suffering from week physical strength subsequent to his advanced age who injected himself with an extract of crushed testicles from guinea pig and dog, which resulted in an improved physical state (252). However, in 2002, Cussons et al. demonstrate that regarding the output of the testicle preparation injected, Brown-Sequard was victim of the placebo effect (61). Later, in 1920, Voronoff executed several chimpanzee-to-human sexual organ xenotransplantations. He performed several testis transplantations believing that the hormones produced by the xenograft would rejuvenate his patients. He also transplanted ovaries of female apes to women in order to treat menopause (13). More surprisingly, he transplanted a woman's ovary into a female chimpanzee that he then inseminated with human sperm and the outcome was unsuccessful (33).

At the beginning of the 20th century, the first organ xenotransplantation began with the mastering of the suturing technique, i.e. the anastomosis. In 1906, Mathieu Jaboulay realised the first true-reported xenotransplantation where he transplanted two patients with kidneys from pig or goat; however both attempts failed due to thrombosis (134). During that time, several animal-to-human kidney transplantations were unsuccessfully attempted; all these failures were directly linked to the absence of immunosuppression. In 1964, Reemtsma performed the most successful chimpanzee-to-human kidney transplantation with the graft surviving 9 month (229). With the numerous failures, the discovery of immunosuppressive drugs and the acceptance of deceased organ donors as a new source of allogeneic organs, the enthusiasm for xenotransplantation vanished and focus went back to allotransplantation. The new xenotransplantation era began in 1984 with the most famous baboon-to-human heart transplantation to a 12-day-old female baby known as "Baby Fae" who died 20 days after surgery (128, 155). Most of the hopes put in xenotransplantation died with Baby Fae and it was only in 1992 that attempts resumed (277).

Currently, clinical pig-to-human trials involving porcine islet cells are performed in New Zealand. Six patients with type I diabetes were transplanted with encapsulated neonatal porcine islet cells secreting insulin. Preliminary results showed

that after transplantation, patients' insulin dose was significantly reduced. One of the patients is said to be insulin-free (Living cell technologies, http://www.lctglobal.com/Products-and-Services/Diabecell/). These findings need to be confirmed and published; however, this recent success demonstrates the great potential of xenotransplantation. For a long period of time, primates were considered the best organ donors for xenotransplantation due to their relatively similar immune system. However, being closely related to human, they represent a higher risk for the transmission of infectious diseases compared to other more distant species. Miniature pigs represent the more suitable source of organ for several reasons. They can be housed in a pathogen-free environment, they are easy to breed and have a bigger number of offspring and their organs share some similarities with human organs regarding size and physiology. Finally, scientists have now a considerable genetic engineering knowledge, which allowed them to create pigs expressing several human complement regulatory proteins (181) responsible for the delay of xenograft rejection.

This approach also presents some unknowns and the most profound obstacle to xenotransplantation remains the immunological rejection of the organ. The potential risk of cross-species infection associated with the use of porcine tissues in humans requires considerable attention. Indeed, xenotransplantation creates a unique situation in which tissues from two species will be in an intimate contact for a long period of time raising the question of possible transfer of pathogens between the graft and the recipient. Most of the research has been focused on donor-derived infections, neglecting host-derived infection with potentially direct consequences for the graft.

3.7 Limits of xenotransplantation

Before xenotransplantation can be regarded as a veritable clinical alternative to allotransplantation, several limitations need to be overcome. Limitations can be classified into two categories: i) biological issues including the immunological and the infectious aspects, and ii) the non-biological issues counting ethical, religious and legal facets. In the following chapter I will discuss the biological issues with a focus on the possible transfer of pathogens between the porcine graft and the recipient.

3.7.1 Classification of xenografts

Xenografts can be classified into two groups regarding the nature of the xenograft and the speed of rejection, including vascularized xenografts (i.e. solid

organs) and non-vascularized xenotransplants (also referred to as free tissue graft) including among them pancreatic islets, bone marrow and neuronal cells. The main difference between the two groups is the time spent between surgery and exposure of the graft to the recipient's blood. Vascularized organs are immediately in contact with the recipient's circulation after transplantation, whereas a free tissue graft will not be exposed until revascularization has occurred, resulting in a faster/immediate rejection for the former. Rejection of vascularized xenografts occurs mainly by humoral mechanisms whereas free tissue xenotransplants are rejected mainly by cellular mechanisms.

In 1970, Sir Roy Calne was the first to described two types of vascularized xenotransplants depending on the relation between the recipient and the donor: concordant and discordant (26). The former consists in transferring organs between two phylogenetically closely related species, for instance nonhuman primate-to-human, whereas the latter describes combination of phylogenetically distant species such as pig-to-human transplantation. A discordant xenotransplantation is characterized by a fulminant rejection occurring within minutes to hours after transplantation whereas a concordant xenotransplantation results in rejection more than one day after surgery.

The different rejection mechanisms described in the following chapter, except for IBMIR, are involved in the rejection of vascularized xenografts.

3.7.2 The immunologic barrier

Following allotransplantation, one hurdle of considerable importance that the graft will encounter in the recipient is the immunological barrier, and in the case of xenotransplantation, the immune response towards the graft, which depends on the phylogenetic distance, will be stronger. Indeed, the more distant the two species are, the stronger the recipient's response will be, which is a big concern regarding pig-to-human transplantation. The humoral and cellular immune systems are involved at different stages of the xenograft rejection and are named: hyperacute rejection (HAR) (147), acute humoral xenograft rejection (AHXR) (222) (also referred to as acute vascular rejection, AVR, or delayed xenograft rejection, DXR), acute cellular xenograft rejection (ACXR) (150) and chronic rejection (257), which are discussed below.

3.7.2.1 Hyperacute rejection (HAR)

HAR characteristics include diffuse interstitial haemorrhage, oedema and thrombosis in capillaries and small vessels (12), leading to the destruction of the xenograft within minutes to hours after surgery. HAR occurs only in vascularized discordant xenotransplantation due to the presence of preformed xenoreactive natural antibodies (XNA) in the discordant recipient responsible for this violent rejection. These antibodies, discovered in 1984 by Galili, are directed against the glycosidic carbohydrate epitope Gal (88). Gal is a terminal disaccharide linked to N-acetyllactosamine on glycoproteins and glycolipids by the enzyme α(l,3)-galactosyltransferase (αGal). This enzyme is expressed in New World monkeys and non-primate mammals such as pigs but is absent in high primates where the gene sequence suggests an inactivation of the enzyme (90); therefore humans and Old World monkey do not express the Gal antigen (89). Anti-Gal antibodies binding to the porcine endothelium, complement deposition, platelet activation and aggregation and coagulation are responsible for the disruption of vascular pEC. Several strategies aiming at either the recipient or the donor have been developed to prevent HAR. One approach consists in depleting the recipient of XNA, which includes: plasmapheresis (which also perturbs the coagulation and the complement cascade components since they are discarded along with the antibody fraction), immunoadsorption (289), perfusion of a porcine "sponge organ" such as a liver or a kidney (14) or administration of decoy carbohydrate polymers (319). However, these techniques only reduce temporally the levels of XNA and titres of these antibodies recur quickly. Other strategies based on genetic engineering of the donor were established: i) the development of transgenic pigs expressing human complement regulatory proteins capable of inhibiting the antibody-mediated activation of the classical complement cascade (40, 69, 181) and ii) the generation in 2002 of αGal knocked-out (αGalKO) pigs by the disruption of the enzyme gene (218).

3.7.2.2 Acute humoral xenograft rejection

AHXR occurs in concordant or in discordant models when HAR is prevented and is for now the main hurdle in xenotransplantation. This second rejection event is characterized by vasculitis, thrombosis, oedema and haemorrhage leading to the xenograft failure in 2 to 5 days. As for HAR, antibodies directed against αGal play a

major role in AHXR; however, xenografts originating from αGalKO pigs and thus not expressing αGal are still susceptible to AHXR, suggesting that non-Gal antibodies are also involved in this delayed rejection (102). This humoral response will cause rejection by fixation and activation of the complement and activation of the endothelium that will in turn induce vascular integrity loss, secretion of cytokines, up-regulation of adhesion molecules on endothelial cells and activate the coagulation pathway (221).

The factors triggering AHXR are currently not well defined and as a consequence treatments to control AHXR are still under investigation. Some treatments include transgenic donors deleted for pro-coagulant genes or overexpressing anti-coagulant factors (60).

3.7.2.3 Acute cellular xenograft rejection

Challenges in avoiding HAR and AHXR have made the characterization of ACXR in discordant xenotransplantation challenging. ACXR generally accompanies AHXR and is characterized by infiltration in the solid organ xenograft of T cells, B cells, natural killer cells (NK cells), neutrophils and macrophages. The prevention of ACXR is based on the induction of T cell tolerance including co-stimulation blockade (27, 123), donor specific transfusion (infusion of donor cells in the recipient) (41, 104) and the induction of mixed haematopoietic chimerism by haematopoietic cell transplantation (the recipient has hematopoietic cells from both his own and the donor origin) (38, 161).

3.7.2.4 Chronic rejection

The changes in the graft associated with a hampered function are called chronic rejection. In xenotransplantation, it is speculated that xenografts surviving all the previous rejection events will develop chronic rejection. For instance, in a concordant aortic hamster-to-rat transplantation model, an accelerated arteriosclerosis occurred (thickening and loss of the elasticity of arteries are considered as a sign of chronic rejection) (247). For now, in order to further investigate chronic rejection in a context of discordant xenotransplantation, HAR, AHXR and ACXR still need to be overcome.

3.7.2.5 Instant blood mediated inflammatory reaction (IBMIR)

This rejection event only occurs after free tissue xenotransplantation. Cellular xenografts are revascularized in and by the recipient, therefore grafts escapes HAR and AHXR (151). Once in the recipient's blood circulation, a high amount of cells is lost due to IBMIR that is characterized by a rapid activation of the coagulation and complement system, infiltration of leukocytes and binding and activation of platelets. All these events will in the end lead to the formation of a thrombus. In 2006, Dwyer et al. demonstrated that mice pancreatic islet cells expressing human CD39 (an ectonucleotidase that degrades the platelet agonist adenosine triphosphate) delayed IBMIR when incubated with human blood (74). In 2012, Wheeler et al. generated a transgenic swine expressing human CD39, however the protective capacity of these cells against IBMIR still needs to be investigated.

3.7.2.6 Coagulation

Coagulation is a tightly regulated process triggered by damages to blood vessel walls and exposure of tissue factor to circulation, triggering the production of thrombin (176). During xenotransplantation, the balance between coagulant and anti-coagulant factors is lost, resulting in thrombosis in the xenograft (59). There are two major incompatibilities between porcine anti-coagulant factor and human coagulant factors:

- between human protein C and porcine thrombomoduline which results in an inefficient conversion by the latter of human thrombin from a pro- to an anti-coagulant form, initiating clotting (148, 233),
- between human factor X (FX) and porcine tissue factor pathway inhibitor (TFPI), resulting in an incapacity of TFPI to inhibit FX (149), although this incompatibility is controversial (164),
- between porcine von Willebrand Factor (vWF) and human platelets where porcine vWF high affinity for human platelet results in a spontaneous aggregation of platelets (251).

There are no pharmacological therapies for the moment because long-term treatment of the recipient is both dangerous and unpractical. The current strategies are

to generate αGalKO transgenic pigs expressing anti-coagulant or anti-thrombotic factors.

3.7.3 The non-immunologic barrier: the risk of zoonosis

Zoonosis refers to the transmission of pathogens from animals (whether wild or domesticated) to humans. Such zoonotic catastrophes previously occurred in history with distressing reports, including: HIV pandemic, Creutzfeldt-Jakob disease, Ebola virus outbreaks, and, more recently, the severe acute respiratory syndrome scare. The potential transmission of pathogens between pig and human is one important concern in xenotransplantation (referred to as xenozoonosis). Although the risk is considered to be smaller with pig organs than with organs from primates, transmission might still occur as a consequence of intensive immunosuppression, and if the pathogen is then transmitted from the recipients to human population, as regards to some of the diseases listed above, it can quickly become a public health issue (309).

In this chapter, I will first discuss about pathogens (focus on viruses) that have been shown to be transmitted from pigs to humans and describe how this transmission risks can be prevented. In the second part I will focus on the infections of the graft by recipient pathogens; this issue has been taken into consideration only recently and is more difficult to prevent.

3.7.3.1 Donor to recipient transmission

It is generally believed that pigs can easily be raised free of infectious agents in a specific pathogen-free (SPF) environment, however it has been shown that transplacental infection of foetuses can occur with a number of viruses, thus periodically, infection of pigs occurs. In this chapter, I will discuss about selected exogenous and endogenous viruses and the potential xenozoonoses risk they present. Because of the lack of specific microbiologic assays to detect pathogen responsible for yet unknown diseases (i.e. linked to unknown clinical syndromes or not related with any identified disease), this section contains an incomplete list. With time and the development of new detection techniques, this list might certainly extend.

3.7.3.1.1 Porcine Influenza virus (Orthomyxoviridae, Influenzavirus A)

Several cases of human infection with porcine flu have been detected where swine influenza virus was isolated from humans (124, 312, 313). Because of several outbreaks, porcine flu is considered a major public health risk. These epidemics include: the Spanish flu (also referred to as the 1918 flu pandemic) causing 50 to 100 million of death that was suspected to be caused by swine influenza virus and more recently the swine influenza outbreak that first manifested in Veracruz (Mexico) and killed an estimated 284,500 people worldwide (65). Swine are very susceptible to human and avian influenza but the infection is usually subclinical or results in a minor disease. In contrast, swine influenza infection in pigs develops rapidly with high fever by 24-36 hours post-infection that lasts 24-48 hours and hyperpnea/dyspnea due to interstitial pneumonia. By 96-120 hours post-infection, fever and clinical signs of respiratory distress diminish. Swine influenza virus is highly contagious and very few herds are free of infection. However, elimination of infection is possible by isolating early weaning from sows and strict biosecurity measures.

3.7.3.1.2 Nipah virus (Paramyxoviridae, Henipavirus)

An outbreak where pigs were suspected to be the intermediate hosts occurred in 1999 in Malaysia among pig farmers causing acute respiratory syndrome and fatal encephalitis in humans (166). Another Nipah virus infection was documented in Singapore in humans working in an abattoir with pigs imported from Malaysia (43). Transmission is thought to occur via respiratory droplets, contact with throat or nasal secretions from the pigs (184) and one can think that contact with tissues of a sick animal could lead to infection. Human-to-human transmission was recorded (114, 244) resulting in high mortality rates, which makes it a serious public health issue. The absence of treatments and the high contagiousness of Nipah virus in pigs make the eradication of herds the best method to control the infection.

3.7.3.1.3 Menangle virus (Paramyxoviridae, Rubulavirus)

Menangle virus was first isolated in Australia in 1997 (35). Reports have demonstrated that two pig framers, later tested positive for Menangle virus antibodies, developed severe influenza-like illness including fever, chills, severe headache, myalgia and a macular rash (35), but both farmers subsequently recovered. To date,

the route of transmission from pigs to humans is still unknown. Adult pigs and new-born do not show any symptoms, but one characteristic of the diseases is a decline in rate and number of live piglets per litter and a large number of mummified foetuses and stillborn. For stillborn, severe degeneration of the brain and spinal cord and grave skeletal/craniofacial defects including arthrogryposis (joint contracture), brachygnathia (abnormal shortness of the lower jaw) and kyphosis (also referred to as roundback) have been observed (171). This virus can be eradicated by disinfection and temporary depopulation of individual units in the pig farm, which was used to control the 1997 outbreak.

3.7.3.1.4 Porcine circovirus (Circoviridae, Circovirus)

Porcine circovirus (PCV) is one of the major pathogens of swine. Infection of humans by PCV is controversial. Some groups did not detect any antibodies in the general population (9, 10) or in veterinarians working with infected pigs (78), whereas Tischer et al. found antibodies to PCV in patients suffering from fever of unknown ethiology (294); however an *in vitro* study demonstrated that infection of human cells by PCV results in a non-productive infection (117). In pigs, the infection named porcine circoviral disease (PCVD) or porcine circovirus associated disease (PCVAD) (previously referred to as post weaning multisystemic wasting syndrome (PMWS)) is characterized by progressive dyspnea and an extreme weight loss in piglets. PCV-free pigs can be obtained by isolating PCV-free pigs from PCV-immune sows (protected as a result of passive acquisition of antibodies) during the first 4 weeks of life and breeding them in a PCV-free environment.

3.7.3.1.5 Encephalomyocarditis virus (EMCV) (Picornaviridae, Cardiovirus)

Although antibodies for EMCV have been demonstrated in human populations (293), there is no report that this virus causes human heart disease. However, Brewer et al. demonstrated that EMCV infects human myocardial cells *in vitro* (23). EMCV is responsible for high mortality in young pigs and reproductive failures in pregnant sows. Piglets suffer from myocarditis and encephalitis and myocardial failure often results in sudden death. An inactivated vaccine for EMCV infection in swine is commercially available and has been shown to be effective. It is also important to minimize the interaction between rodents and swine (rodents are naturally infected

with EMCV and are believed to be the principal reservoir of the virus (1)) and to prevent contact with pigs either directly or indirectly via contaminated food or water.

3.7.3.1.6 Porcine coronavirus (Coronaviridae)

In Japan, 5% of human milk samples collected from mothers were tested positive for the presence of antibody against porcine transmissible gastroenteritis coronavirus (TGEV) (292). Four coronaviruses have been detected in pigs, including TGEV, porcine respiratory coronavirus, porcine epidemic diarrhea virus and hemagglutinating encephalomyelitis virus. From all these four circoviruses, TGEV is the most common and the most studied (242) and is the only coronavirus that has been suggested to be transmitted to humans (292). Swine infected with TGEV have reduced capability for digesting food and die from dehydration. Preventive measures for negative herds include maintaining a closed herd and implementing strict biosecurity practices.

3.7.3.1.7 Herpesvirus (Herpesviridae)

In this paragraph, three porcine herpesviruses will be discussed: pseudorabies virus, porcine lymphotropic herpesvirus and porcine cytomegalovirus. Although to date no transmission from pig to human has been reported, these viruses cause persistent infections and could represent a xenozoonotic risk.

- Pseudorabies (PRV) (α-herpesvirus, Varicellovirus)

Reports of human infection are limited and are based on seroconversion rather than virus isolation. In pigs PRV infection is usually asymptomatic but it can cause severe respiratory disease, neurological disease, and abortions. Piglets are more susceptible and suffer from severe respiratory disease with high mortality. In 2004, Hayashi et al. demonstrated that the anti-Gal antibodies in human serum are important for neutralizing PRV from pigs expressing Gal (118). The remaining question would then be whether humans would be able to neutralize PRV from Gal-KO pigs? Although the ability of PRV to cause latent infection complicates its eradication and control, it is possible to prevent exposure of a negative herd to PRV through strict quarantine and isolation procedures.

- Porcine lymphotropic herpesvirus (PLHV) (γ-herpesvirus, Rhadinovirus)

To date, three PLHV have been discovered: PLHV-1 and PLHV-2 in 1999 (77) and PLHV-3 (more distant genetically from PHLV-1 and PLHV-2) in 2003 (44). In pigs immunosuppressed and subjected to allogeneic hematopoietic stem cell transplantation, PLHV-1 is associated with a porcine lymphoproliferative disease of high mortality (103, 127) which resembles the post-transplantation lymphoproliferative disease (PTLD) associated with Epstein-Barr virus infection in humans. Santoni et al. demonstrated that PLHV-1 is able to upregulate KSHV promoters and transactivate EBV promoters *in vitro* (243), which suggests that molecular interactions between human and porcine gammaherpesviruses might occur *in vivo*. The mode of transmission of PLHV is still unknown making breeding of PLHV-free herds challenging as for instance simple weaning of piglets is not sufficient (195). Tucker et al., suggested that PLHV-free piglets can be obtained via ceasarian-derived and barrier-reared breading procedures (297).

- Porcine cytomegalovirus (PCMV) (β-herpesvirus, Cytomegalovirus)

Severity of PCMV infection depends on the age and the immune status of the pig and immunity of the sows. In adult pigs, infection is usually unapparent or can present subclinical signs (respiratory signs), whereas in piglets and newborn pigs, infection induces a generalized infection that is often fatal (76). In a pig-to-baboon model of xenotransplantation, Mueller et al. demonstrated that PCMV was upregulated in 83% of the animals and PCMV infection was associated with ureteric necrosis of one xenograft (Mueller, 4734-40). The susceptibility of PCMV to the current antiviral therapy is controversial since one team reported a reduced susceptibility of PCMV to these agents (196) whereas another team reported an inhibition of PCMV replication (84). No vaccine or specific treatment for PCMV is available but early weaning of newborns within the first two weeks after birth and isolation of the piglets from other individuals can achieve exclusion of PCMV in herds.

3.7.3.1.8 Porcine endogenous retroviruses (PERV) (Retroviridae, Gammaretrovirus)

PERV are inheritable viruses and therefore, in contrast to exogenous viruses, cannot be excluded from pig herds. While PERV particles were detected in pig cell lines derived from leukemias (193), no pig disease has been linked to PERV infection. Three classes of replication competent PERV, PERV-A, PERV-B and PERV-C have been described (163, 286) and human-tropic PERV are a recombination of PERV-A and PERV-C (209). Although it was recognized by Patience et al. that PERV can be transmitted to human cells and results in a productive infection *in vitro* (212), no *in vivo* transmission has been reported in human even 8 years after receiving pig islet cells (298). Several strategies have been developed in order to minimize the risk of PERV transmission, including: selection of PERV-C-free animals to prevent recombination of PERV-A and PERV-C; selection of animal expressing very low levels of PERV-A and PERV-B by discriminating high producers, whose PBMC produce high amounts of PERV upon stimulation, from low producers (285); development of vaccine (82) and finally inhibition of PERV expression by using PERV-specific small interfering RNA (siRNA) as well as short hairpin RNA (shRNA) and retroviral vectors (70, 143, 189).

3.7.3.2 Recipient-to-donor transmission

In this chapter, I will discuss about human viruses that have been reported to have interactions with porcine cells. The concerns about the infectious risks have been focused predominantly on the transmission of pathogens from animal-to-human, i.e. pig-derived infections, and the possibility of human virus infection and destruction of transplanted pig organs have been appreciated only recently, which renders the list below non exhaustive.

3.7.3.2.1 Adenoviruses (Adenoviridae)

Humans infected with adenoviruses display a wide range of responses, from no symptoms at all to acute respiratory infections with fever and conjunctivitis referred to as epidemic keratoconjunctivitis (EKC). In 1957, Guerin et al. demonstrated for the first time that human adenoviruses (hAdV) infect porcine kidney cells *in vitro* (113). More recently, three groups described efficient hAdV replication of a few hAdV types (1, 4, 5, and 17) in immortalized porcine cell lines and primary

cell cultures of several organs and tissues resulting in complete cytopathic effects (108, 138, 160). Swine infected experimentally with hAdV develop lesions in the respiratory tract but did not show clinical signs (17). Several studies demonstrated that infection of human endothelial cells with hAdV induces an upregulation of adhesion molecules and cytokines secretion, causing enhanced leukocyte adhesion and transmigration (226, 287). Since interaction of human neutrophils with porcine endothelium can cause damages upon stimulation (28), one might speculate that if similar modulations occur in hAdV-infected porcine endothelial cells, an increased adhesion of human neutrophils to porcine endothelium may then initiate injury to the porcine vessel walls and cause xenograft rejection.

3.7.3.2.2 Coxsackievirus B5 (CVB-5) (Picornaviridae, Enterovirus)

Humans infected with CVB-5 present symptoms including fever, headache, sore throat, gastrointestinal distress as well as chest and muscle pain. Coxsackieviruses B are considered to be associated with the pathogenesis of type I diabetes (T1D), indeed epidemiological studies have demonstrated that coxsackievirus infection is a very frequent event in patients suffering from T1D (92). In 1957, as for adenoviruses, Guerin demonstrated for the first time that porcine kidney cells are susceptible to CVB-5 *in vitro* (113). Later, Myers et al demonstrated that i) porcine pancreatic islet cells are susceptible to CVB-5 (200), which poses a special concern for islet xenotransplantation and that ii) CVB-5-infected porcine pancreatic islet cells can transmit the virus to immunosuppressed mice recipients (201). The role of CVB-5 in clinical xenotransplantation is unclear, thus further studies to investigate CVB5 infection in mice or nonhuman primates followed by xenotransplantation of uninfected pig islets are needed to analyse host-to-graft xenozoonosis as well as prophylaxis and antiviral therapies for use in xenotransplantation.

3.7.3.2.3 Hepatitis C virus (HCV) (Flaviviridae, Hepacivirus)

HCV acute infection is often asymptomatic, in contrast to chronic infection which can lead to cirrhosis or hepatic cancer many years later. In some cases, individuals with cirrhosis will develop liver failure, liver cancer or life-threatening oesophageal and gastric varices. Two groups demonstrated the susceptibility of porcine cells to HCV *in vitro* (144, 254). In both cell lines, HCV established a persistent infection and lysis of infected cells released in supernatants viral particles

which were able to infect fresh cultures (254). The difficulty to establish HCV infection in cell culture system renders challenging the assessment of infectivity in a cross-species model, but studies suggest an infective potential of HCV in porcine cells.

3.7.3.2.4 Influenza virus (Orthomyxoviridae, Influenzavirus A)

Symptoms of infection with influenza virus include high fever, extreme fatigue, body ache and cough. Because of their capacity to be infected by influenza viruses from different species (swine, human and avian), the concept of "mixing vessels" was proposed for pigs, because viruses can reassort in swine and give rise to new, potentially more pathogenic strains (174). As written in chapter 3.7.3.1.1, swine are very susceptible to human influenza virus but the infection is usually subclinical. In contrast, an *in vitro* study demonstrated that infection of porcine lung endothelial cells with human influenza virus (H3N2) resulted in cytopathic effect appearing 18 hours post infection and expression of viral proteins (255). Later, Van Poucke et al. reported that the pattern of human influenza replication in porcine cells (from the entire porcine respiratory tract) resembles that of swine influenza virus (299 7:38). These studies suggest that human influenza can induce damage to porcine graft, thus a treatment must be established for xenograft recipients.

3.7.3.2.5 Herpes simplex virus 1 and 2 (HSV-1, HSV-2) (Herpesviridae, Simplexvirus)

Symptoms of herpes simplex virus infection include watery blisters in the skin or mucosa of the mouth, lips or genitals, and infection can also have a more severe outcome with devastating encephalitis (HSV-1). In a first study, it was reported that porcine testis cells were defective for HSV-1 and HSV-2 replication because of a defective entry of these viruses. The entry of HSV into porcine testis cells is the limiting step of infection (281). However, when porcine cells were expressing the herpes virus entry mediator (HVEM) protein after stable transformation, HSV binding, entry, replication, and spread was reported (215). Recently, Kim et al. demonstrated that porcine kidney epithelial cells are permissive to HSV-1 and HSV-2 infection, which challenges the previous statements (145). Since no further data is available, prophylaxis of HSV infection in xenotransplantation trials should be considered.

3.7.3.2.6 Human cytomegalovirus (HCMV) (Herpesviridae, Cytomegalovirus, β-herpesvirus)

Infection of HCMV is mainly asymptomatic in immunocompetent hosts and it can become life threatening in immunodeficient hosts. As for HSV, HCMV was reported to be species specific, however in 2001 Degré et al. demonstrated for the first time that a clinical isolate enters and produces viral proteins in porcine endothelial cells. Porcine cells were also shown to release infectious viral particles since the supernatant of infected cells could infect a second generation of cells (68). More recently, Millard et al. extended these findings by using endothelial cells from different anatomical origins as well as different HCMV strains. They reported atypical cytopathogenic effect, which depends on the vascular origin of the cells, a productive infection although less efficient when compared to human endothelial cells and induction of apoptosis (185). The group of Seebach and Schneider reported that HCMV entry into porcine endothelial cells is sufficient to induce phenotypic changes including an increased surface expression of adhesion molecules such as E-selectin and vascular cell adhesion molecule 1 (VCAM-1), both critical mediators of leukocyte recruitment. An increased expression and shedding of adhesion molecules was paralleled by enhanced human leukocyte chemotaxis and adhesion to infected pEC cultures (94). The overall effect mediated by HCMV infection of porcine endothelial cells seems to have the potential to cause transplantation-associated vasculopathy, therefore, a prophylactic treatment approach may be necessary.

4 Aims of the study

Transplantation has emerged as a valid therapy to treat patients with end-stage organ failure. However, a major limitation is the increasing shortage of donor organs. Xenotransplantation using pig organs is a promising experimental approach to increase availability of organs. Clinical pig-to-human trials involving islet cells are currently performed. Infection or reactivation of HCMV is the most important infectious complication post-transplantation since HCMV infection has been associated with allograft rejection and the consequences of HCMV cross-species infection of porcine xenografts are still unknown. If xenotransplantation proceeds to the clinical stage, understanding the potential role of cross-species CMV infection and prevention of porcine cells infection will be a crucial step to ensure safety and optimal functioning of the xenograft.

The molecular basis responsible for HCMV entry into porcine cells remains uninvestigated. Given the profound effect of pEC infection with HCMV, knowledge of this mechanism is required for interventional strategies. The following aims were outlined for this thesis:

Aim #1: Analyze the involvement of know human entry receptors in HCMV entry into pEC and compare them with hEC.

Aim #2: Determine the involvement of endocytosis, the endosomal pH acidification and the cellular cytoskeleton in HCMV entry.

Aim #3: Analyze the involvement of host species in HCMV nuclear translocation and initiation of replication.

5 Material and methods

This section includes material and methods from the manuscript in preparation and supplementary protocols.

5.1 Cells

SV40-immortalized porcine aortic and bone marrow-derived EC lines (PEDSV.15 and 2A2, respectively) were established in our laboratory and were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate (Invitrogen), 2 mM L-glutamine, non-essential amino acids (1x) (Invitrogen), essential amino acids (1x) (Invitrogen), and 20 mM HEPES (Invitrogen). Primary human aortic and microvacular EC (HAEC and HMVEC, respectively) were obtained from Lonza and were cultured in complete EGM-2 medium following the manufacturer's protocol. The human embryonic lung fibroblast cell line (MRC-5) was obtained from ATCC and cultured in DMEM medium supplemented with 10% FCS. All cell preparations were tested for mycoplasmas by 4, 6-diamidino-2-phenylindole (DAPI) staining.

5.2 Virus

HCMV endotheliotropic (TB40/E) and fibrotropic (TB40/F) strains were derived from a bone-marrow transplant recipient by 22 passages in EC and fibroblasts, respectively (265). For preparation of virus stocks, MRC-5 were infected with TB40/E or TB40/F at a multiplicity of infection (MOI) of 0.5. Supernatants of infected cultures were harvested at 7 days post infection (dpi) and cleared of cellular debris by a 10 minute (min) centrifugation at 2,500 g. Virus stocks were partially purified by an ultracentrifugation step over a 15% sucrose cushion (50mM Tris-HCl, 12mM KCl, 5mM Na_2EDTA) at 20,000 rpm for 90 min at 4°C using a SS-34 rotor (Beckman Coulter). Virus aliquots were stored at -80°C. The infectious titer was determined by TCID50 assays in MRC-5 seeded in 96-well plates (177). All virus preparations were tested for mycoplasmas by DAPI staining.

5.3 Reagents and antibodies

Heparin, bafilomycin A1, chlorpromazine, dynasore, EIPA (5-N-ethyl-N-isoproamiloride), filipin, cytochalsin D, nocodazole and acridine orange were

obtained from Sigma. The following primary antibodies were used in this study: rabbit anti-β1 integrins (Millipore for Western blot), APC conjugated mouse anti-β1 integrins (Thermo Scientific for flow cytometry, clone MEM-101A), mouse anti-αV integrin (Santa Cruz, clone P2W7) and mouse anti-β3 integrin (Santa Cruz, clone BV4), PE conjugated mouse anti- PDGFRα (Santa Cruz clone 16A1 for flow cytometry), rabbit anti-PDGFRα (Santa Cruz for Western blot), AF488 conjugated mouse anti-EGFR (Millipore for flow cytometry, clone LA1), rabbit anti-EGFR (Santa Cruz for Western blot), mouse anti-viral glycoprotein B (gB) (Abcam), mouse anti-CMV IE72/IE86 (Argene), mouse anti-human β-actin (Sigma), mouse anti-LAMP2 (BD Pharmingen), mouse anti-EEA1 (BD Transduction Laboratories).
The following secondary antibodies were used in this study: Alexa Fluor 488 conjugated goat anti-mouse (Molecular probes), Cy3-conjugated goat anti-mouse (Jackson ImmunoResearch) and PE conjugated goat anti-mouse (BD PharMingen).

5.4 Flow cytometry analysis

Cells grown in 6-well plates (200,000 cells/well) were incubated for 5 min with PBS-2nM EDTA, washed and stained for 30 min at 4°C with PE, APC or Alexa Fluor 488-conjugated monoclonal antibody (mAb) to PDGFRα, β1 integrins and EGFR, respectively. The PE or APC-conjugated MOPC21 (BD Pharmingen) and the Alexa Fluor 488 conjugated normal mouse IgG (Millipore) were used as isotype-matched controls. The fluorescence intensity was measured by using a FACSCanto (BD Biosciences) and analysis was performed using FlowJo software (Tree Star).

5.5 Membrane extractions

Cells grown in 10 cm Petri dish (300,000 cells/Petri dish) were washed twice, scrapped in ice cold PBS and centrifuged 10 min at 2,700 rpm at 4°C. The pellet was resuspended in homogenization buffer (500µL/Petri dish, 0.25M sucrose, 0.1M K_2HPO_4, 1mM EDTA) containing 1X protease inhibitors (Roche) and sonicated until the cells went from white, cloudy to an opalescent solution. Thereafter, cells were centrifuged 15 min at 2,000 rpm at 4°C and the supernatant was centrifuged at 55,000 rpm for 1 hour at 4°C. The supernatant containing the cytoplasmic proteins was saved and the pellet containing the membrane proteins was resuspended in SDS sample buffer lacking bromophenol blue. Detection of entry receptors by Western blot was

performed from 50μg of proteins and purity of the extraction was assessed by detection of tubulin.

5.6 Detection of MxA proteins after Small interfering RNA transfection

Human EC seeded in 24-well plates (50,000 cells/well) were left untransfected or transfected the next day with 31.25ng of non-targeting control siRNA (Ctrl) (Santa Cruz) using standard Lipofectamine 2000 protocol (Invitrogen). Alternatively, human EC were seeded in 24-well plates and transfected with 12.5ng of SiRNA on the same day using standard Hiperfect reagent protocol (Qiagen). 24 hours later cells were scraped and lysed (as described below) and the expression of the interferon inducible protein MxA was analysed by Western blot. β-actin was used as a loading control.

5.7 Small interfering RNA assay

Human EC were transfected in 24-well plates (50,000 cells/well) with 12.5ng of either the siRNA to human β1 integrins, PDGFRα or the Ctrl siRNA (Santa Cruz) using standard Hiperfect reagent protocol (Qiagen). Twenty-four hours after transfection, siRNA-transfected cells were TB40/E- or mock-infected with an MOI of 1. Porcine EC seeded in 24-well plates (70,000 cells/well) were transfected the next day with 31.25ng of the siRNA listed above using standard Lipofectamine 2000 protocol (Invitrogen). Twenty-four hours after transfection, siRNA-transfected cells were infected with TB40/E at an MOI of 1 for PEDSV.15 cells and 0.5 for 2A2 cells or with TB40/F at an MOI of 0.5 and 0.3 for PEDSV.15 and 2A2 cells, respectively. All four cell types were then scraped and lysed (as described below) 24 hours post infection (hpi). The expression levels of PDGFRα, β1 integrins and the viral immediate early (IE) proteins were analysed by Western blot. β-actin was used as a loading control.

5.8 Cell proliferation analysis

Human EC and porcine EC seeded in 100μl (serum-free medium) per well of a 96-well plate (3,000 cells/well) were incubated with different inhibitors for 2.5 hours. Cells were then washed with PBS to discard the drugs and incubated for another 24 hours in fresh medium with serum. Cell proliferation was quantified with an

AlamarBlue® (Invitrogen) assay following provider's information. Briefly 10µl of AlamarBlue® were added in each well and incubated for another 4 hours at 37°C and the absorbance of AlamarBlue® was read with a spectrophotometer (Victor). The mean of four independent experiments is shown.

5.9 Cell cycle progression analysis

Human and porcine EC seeded in 6-well plates (200,000 cells/well) in serum-free medium were treated for 2.5 hours with the different inhibitors, washed with PBS to discard the drugs and incubated for another 6.5 hours at 37°C in fresh medium with serum. Cells were then trypsinized, washed twice with cold PBS by resuspending and spinning down (2,000 rpm, 5min). After the last centrifugation, supernatant was discarded and cells were fixed with 70% ice-cold ethanol and incubated overnight at 4°C. The next day, cells were centrifuged 5 min at 3,000 rpm, the pellet was stained for 30 min at 37°C in the dark under gentle shacking with a staining solution (PBS, 0.3mg/ml of RNAseA, 0.3mg/ml of propidium iodide). After centrifugation (5 min, 3,000 rpm) cells were resuspended in 100µl of the staining solution and analysed by flow cytometry within 24 hours. The fluorescence intensity was measured by using a FACSCanto (BD Biosciences) and analysis was performed using FlowJo software (Tree Star). One representative experiment out of three is shown.

5.10 Binding assay

Human EC and porcine EC grown in 24-well plates (50,000 cells/well) in serum-free medium were treated for 1 hour at 4°C with different inhibitors. Cells were then infected with TB40/F at an MOI of 5 for 1.5 hours at 4°C in presence of the drugs. As a control, TB40/F was preincubated with 100 µg/ml of heparin for 1 hour at 4°C, and then added to the cells and incubated for an additional 1.5 hours at 4°C. Cells were then washed extensively with ice-cold PBS and lysed as described below. The accumulation of the tegument protein pp65 was then analysed by Western blot. β-actin was used as a loading control.

5.11 HCMV infection inhibition assay

Cells grown in 96 well-plates (9,000 cells/well) and in serum-free medium were treated with nontoxic doses of various inhibitors for 1 hour at 37°C before being

inoculated with TB40/E (MOI of 1 for PEDSV.15 cells and human EC and MOI of 0.5 for 2A2 cells) or TB40/F at an MOI of 0.5 (PEDSV.15 cells) or 0.3 (2A2 cells) for 1.5 hours at 37°C. Thereafter, non-internalized viral particles were discarded by extensive washes with PBS. Cells were then incubated for 6.5 additional hours at 37°C. After a fixation step of 10 min in a 80% acetone solution, cells were incubated for 1 hour at 37°C with the primary antibody anti-IE (1/500), subsequently incubated at 37°C for 1 hour with a Cy3-conjugated goat anti-mouse antibody (1/500), and counterstained with DAPI. After each step, the cells were washed three times in PBS. The cells were analysed with an IX71 microscope (Olympus). The analyses were performed at 20-fold magnification on eight distinct fields. The infection rate was then calculated as the mean value of the number of IE positive cells counted on the eight distinct fields per condition divided by the mean value of the total number of counted cells and multiplied by 100. The mean of four independent experiments is shown, otherwise specified in the legends.

5.12 Measurement of acridine orange intensity after bafilomycin A1 treatment

Human EC and porcine EC seeded in 96-well plates (9,000 cells/well) in serum-free medium were left untreated or were treated with bafilomycin A1 (2 and 20nM) for 1 hour. Cells were then incubated with 5 μg/ml of acridine orange diluted in Hank's Balanced Salt Solution (HBSS) for 10 min. The acridine orange was removed by three washes with PBS and fluorescent pictures were obtained using an IX71 microscope.

5.13 Co-localization analysis

Human EC and porcine EC grown in shell vials (Milian) (50,000 cells/shell vial) coated with bovine fibronectin (Sigma) were infected with TB40/E or TB40/F at an MOI of 5 for 1 hour at 4°C, washed with cold PBS and shifted to 37°C for the indicated times. Subsequently, cells were washed three times with PBS and fixed with 3% paraformaldehyde (Sigma) for 10 min at room temperature, permeabilized 5 min with PBS-Triton 0.2%, followed by extensive washes in PBS. Cells were blocked for 30 min at room temperature with PBS-10% FCS. Glass coverslips staining was performed in four steps, each step consisting of a 1-hour incubation at 37°C followed by three washes of 5 min with PBS: 1) staining with a primary antibody to gB

(1/400), 2) staining with a goat anti-mouse antibody labelled with Cy3 (1/300), 3) staining with a primary antibody to EEA1 (1/300) or LAMP2 (1/400), 4) staining with a goat anti-mouse antibody labelled with Alexa Fluor 488 (1/300). Cells were then counterstained with DAPI (1/10,000) and washed with PBS. Glass coverslips were mounted with ProLong® Gold (Invitrogen) and examined under a Leica SP5 confocal microscope. Colocalization of red and green signal was determined, using the colocalization Test plugin JACoP from ImageJ to calculate Pearson's coefficient. The mean of Pearson's coefficient was calculated from three independent experiments.

5.14 Analysis of nuclear translocation of the tegument protein pp65

Human EC and porcine EC seeded on glass coverslip in shell vials (70,000 cells/well) were infected with TB40/E or TB40/F at an MOI of 1 for different periods of times. Cells were then washed and fixed with a mixture of ice-cold ethanol/acetone (2:1) for 10 min, washed with PBS and let air-dried. Coverslips fixed to a slide were incubated for 1 hour at 37°C with the primary antibody anti-pp65 (1/500), subsequently incubated at 37°C for 1 hour with an AlexaFluor 488-conjugated goat anti-mouse antibody (1/300), and counterstained with Evans Blue cytoplasmic staining. After each step, the cells were washed three times in PBS. The cells were analysed with an IX71 microscope and the analyses were performed at 100-fold magnification.

5.15 Analysis of the tegument protein pp65 accumulation in TB40/E- and TB40/F-infected EC

Human EC and porcine EC seeded in 24-well plates (70,000 cells/well) were infected with TB40/E or TB40/F at an MOI of 1 for different periods of time. Cells were then washed and lysed as described below. The accumulation of the tegument protein pp65 was then analysed by Western blot. β-actin was used as a loading control.

5.16 Time course of infection

Human EC and porcine EC grown in 48-well plates (50,000 cells/well) were infected with TB40/E or TB40/F at an MOI of 1 for 1h at 4°C, washed with cold PBS and then shifted to 37°C for different periods of time. EC were washed and treated with trypsin-EDTA (0.25% trypsin and 5 mM EDTA) for 5 min to remove the bound but non-internalized virus, washed, and total DNA was extracted following the manufacturer's specifications (Qiagen, DNeasy Blood & Tissue Kit). DNA

concentration was measured with the Nanodrop and 30ng of total DNA was quantified using specific primers and probes for a 62 bp region of the glycoprotein B gene (UL55) as previously described (324). Amplification was performed in a Real-Time thermocycler (iQ5 Cycler; Bio-Rad) as follows: 13min 30s at 95°C and 50 cycles of 15s at 95°C and 60s at 60°C. Each reaction was done in duplicate, and each point represents the average +/- standard deviation (SD) of one representative experiment.

5.17 Analysis of the initiation of replication in TB0/E- and TB40/F-infected EC

Human EC and porcine EC seeded in 24-well plates (70,000 cells/well) were infected with TB40/E or TB40/F at an MOI of 1 for different periods of time. Cells were then washed and lysed as described below. The expression of the IE proteins was then analysed by Western blot and β-actin was used as a loading control.

5.18 Analysis of the involvement of FAK in HCMV entry into human EC

Human EC seeded in serum-free medium in 24-well plates (70,000 cells/well) were infected with TB40/E or TB40/F at an MOI of 3 for different periods of time (10, 30 and 60 min). As a positive control, cells were incubated for 5 min with PBS supplemented with 20% FCS. Cells were then washed and lysed as described below. The expression of FAK (Santa Cruz, dilution 1/200) and the phosphorylated form of FAK (Invitrogen, dilution 1/1000) was analysed by Western blot and β-actin was used as a loading control.

5.19 Western blot analysis

Cultures grown in 24 well plates were scraped and lysed directly in SDS sample buffer (63 mM Tris HCl, 10% Glycerol 2% SDS, 0.0025% Bromophenol Blue, pH 6.8). Protein samples were separated by electrophoresis on a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Hybond-P, GE Healthcare). After blocking with 5% non-fat dried milk (Bio-Rad) in 2% Tween/TBS, membranes were incubated overnight with primary antibody at 4°C, followed by a 1-hour incubation with a horseradish peroxidase-conjugated secondary antibody (GE

Healthcare, 1/5000). The targeted proteins were revealed by enhanced chemiluminescence using the ECL reagent (GE Healthcare). One representative experiment out of three independent experiments is shown, otherwise specified in the legends.

5.20 Statistical analysis

All values are reported as mean values ± SD. Statistical analysis were performed using the unpaired Student t test with Prism GraphPad software, with statistically significant differences set as: *p< 0.05, **p < 0.01,***p < 0.001,****p < 0.0001.

6 Results

The results section of this thesis is divided into 3 main parts corresponding to the three main steps of HCMV entry into target cells:

- The first part focuses on the role of the different receptors,
- The second part investigates mechanisms of virus internalization by target cells,
- The third part elucidates nuclear translocation of the virus following internalization and the initiation of viral replication.

A manuscript has been prepared and submitted for publication.

6.1 Molecular basis of HCMV entry into porcine endothelial cells

6.1.1 Cell surface expression of HCMV entry receptors

To enter human cells (mainly fibroblasts, epithelial and endothelial cells), HCMV utilizes different cellular receptors such as PDGFRα and EGFR and co-receptors including β1, αV and β3 integrin subunits (34, 81, 272, 307, 308). Data on the requirement of these receptors and co-receptors is controversial (131, 301), and dependent on the cell type studied. HAEC, HMVEC, PEDSV.15 and 2A2 cells were first analysed by flow cytometry for the surface expression of β1, αV, β3 integrins, EGFR and PDGFRα. All four cell types exhibited high levels of β1 integrins and no EGFR, whereas PDGFRα and αV and β3 integrin subunits were detected only on hEC, with a weaker β3 integrin subunit expression observed on HAEC compared to HMVEC (Fig. 4).

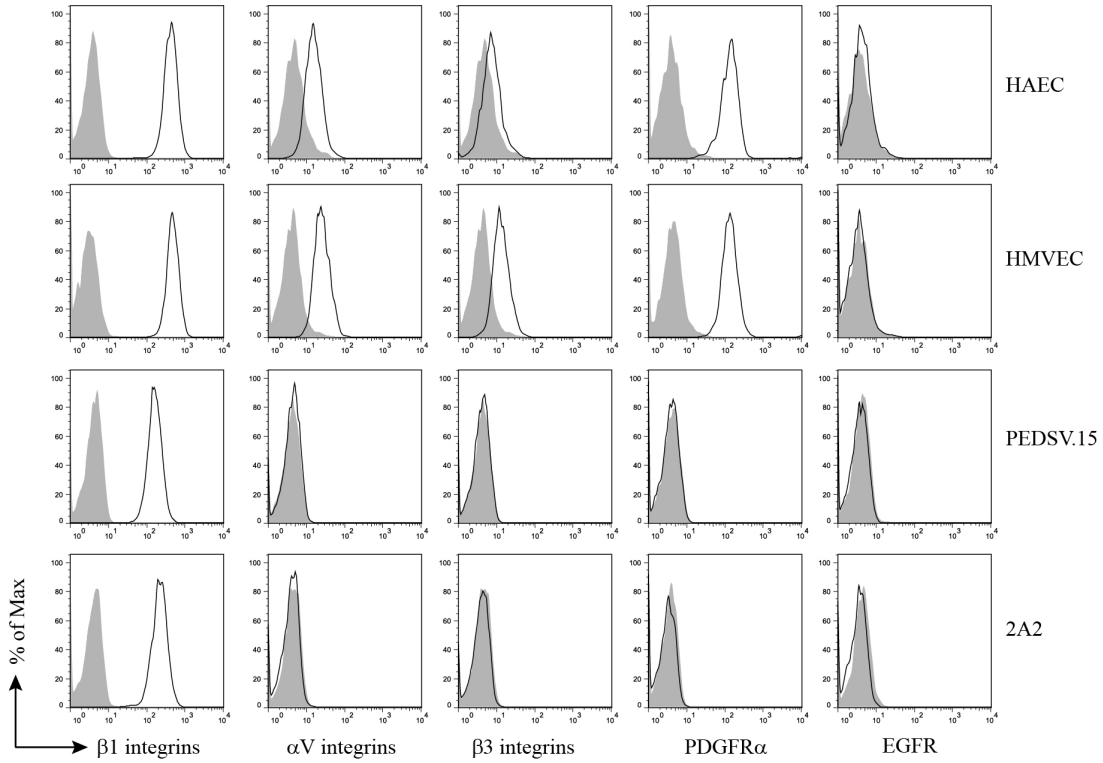

Fig. 4: Comparative surface expression of HCMV entry receptors and co-receptors on human and porcine EC. hEC and pEC were harvested and the surface expression of HCMV entry receptors was analysed by flow cytometry (Grey filled histograms: control isotype, Black line histograms: indicated receptor). HAEC: human aortic EC, HMVEC: Human dermal microvascular EC, PEDSV.15: porcine macrovascular aortic EC, 2A2: porcine microvascular bone-marrow derived EC. One representative experiment is shown out of three independent experiments.

In the absence of antibodies specific for porcine entry receptors, flow cytometry analysis for PDGFRα, β1 integrin subunit and EGFR was conducted with anti-human monoclonal antibodies expected to be cross-reactive, based on the protein sequence. To determine if the lack of detection of entry receptors on pEC surface was due to missing cross-reactivity of these antibodies, Western blot analysis of pEC total extracts was performed using anti-human polyclonal antibodies (Fig. 5A). PDGFRα was detected in both types of pEC whereas EGFR was only detected in 2A2 cells. In order to determine the location of these receptors, we conducted membrane extraction and analysed the expression of PDGFRα and EGFR in the different fractions. PDGFRα was detected in the membrane fraction of both pEC whereas EGFR was detected only in the membrane fraction of 2A2 cells (Fig. 5A). In order to exclude the possibility of the expression of a truncated but functional EGFR on PEDSV.15 cells, cells were stimulated with EGF (100μg/ml) for 10 min and the activation of EGFR signaling pathway was assessed by the detection of the phosphorylated form of Akt in total extracts by Western blot. The treatment of PEDSV.15 cells with EGF failed to activate the EGFR signaling pathway represented by the absence of the phosphorylated form of Akt although the non-phosphorylated form was detected, whereas the stimulation of 2A2 cells induced the phosphorylation of Akt, which suggests that PEDSV.15 cells do not express EGFR (data not shown).

Concerning αV and β3 integrin subunits, both antibodies were anti-human polyclonal antibodies. In order to exclude any incompatibility of the antibodies with the flow cytometry technique resulting in the absence of detection of αV and β3 integrin subunits, their expression was as well analysed by Western blot in the total extracts, cytoplasmic and membrane fractions of pEC. αV integrin subunit was detected in the total extract and the membrane fraction of 2A2 cells and to a lesser extend in the total and membrane fractions of PEDSV.15 cells. A weak expression of β3 integrin subunit was detected in the membrane fraction of 2A2 cells but was absent in total and membrane extracts of PEDSV.15 cells (Fig. 5B).

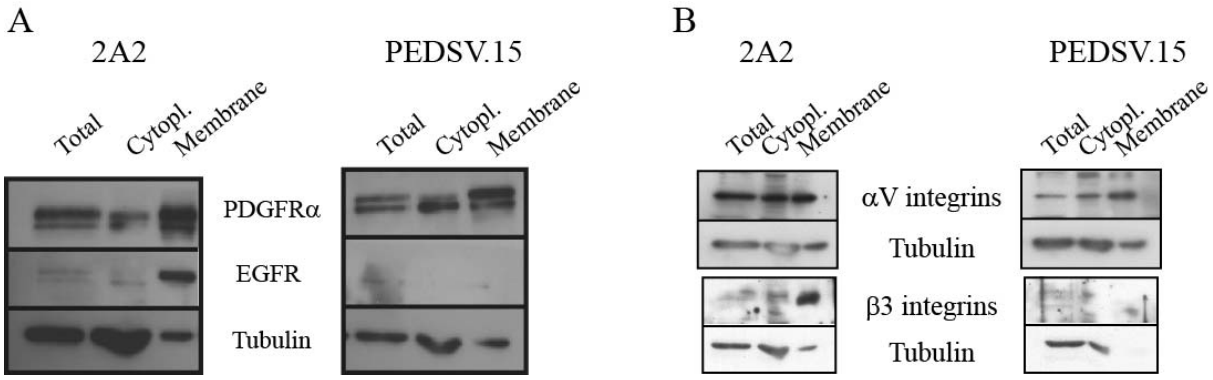

Fig. 5: Localisation of HCMV entry receptors and co-receptors in pEC by Western blot. pEC membrane fractions were prepared and the extracts were analysed for the expression of **(A)** PDGFRα, EGFR and **(B)** αV and β3 integrin subunits. Total: total extract, cytopl.: cytoplasmic fraction, Mb.: membrane fraction. One representative experiment out of two independent experiments is shown.

All together, these results show that similarly to hEC, pEC express a panel of HCMV entry receptors and co-receptors. The next step was then to assess whether HCMV is utilizing these receptors to enter pEC, similarly to the entry into hEC.

Table 3: Table recapitulating the expression of the different receptors and co-receptors on hEC and pEC.

	2A2	**PEDSV.15**	**HMVEC**	**HAEC**
PDGFRα	yes	yes	yes	yes
EGFR	yes	NO	NO	NO
αV integrins	yes	yes	yes	yes
β1 integrins	yes	yes	yes	yes
β3 integrins	yes	NO	yes	yes

6.1.2 Involvement of PDGFRα and β1 integrin subunit in HCMV entry into pEC

First, proper transfection conditions, i.e. non-interferon (IFN) inducing conditions were set up for hEC. The liposomal transfection reagent used for pEC transfection, lipofectamine2000, has been reported to induce, in human cells, Type I interferon (IFN-I) and its inducible proteins (165). Type I IFNs are a highly regulated defense system against viral infection (197) and once produced, they induce an up-regulation of direct antiviral effectors including amongst others the myxovirus protein A (MxA) (5, 99). The literature has reported that high levels of type I IFN are critical early defense mechanisms against CMV infection (111, 178, 179). In my setting, transfection with lipofectamine2000 resulted in the production of MxA protein by

HAEC in irrelevant control short interfering RNAs (SiRNA)-transfected hEC but not in untransfected cells (Fig. 6A), preventing subsequent HCMV infection revealed by the absence of IE proteins in HAEC transfected cells 24 hpi (data not shown). Therefore, hEC were transfected with Hiperfect (Qiagen), another transfection reagent, suggested to lack the IFN inducing effect because of the reduced amount of SiRNA needed for transfection compared to lipofectamine2000. After transfection of HAEC with a control SiRNA, no MxA protein was detected showing that in these conditions, type I IFN is not or weakly induced by the transfection reagent (Fig. 6B).

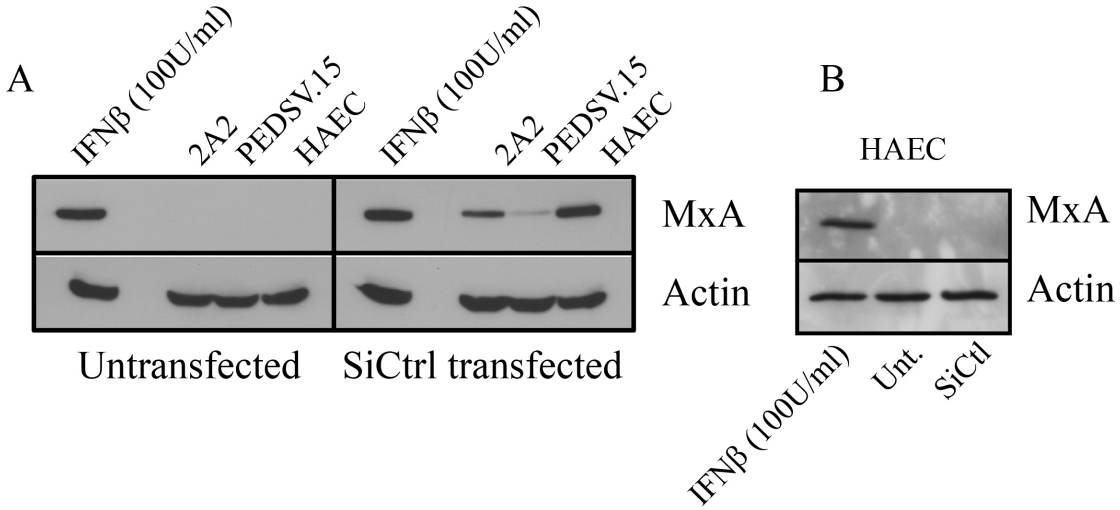

Fig. 6: Induction of MxA protein expression in SiRNA transfected EC. (A) PEDSV.15, 2A2 cells and HAEC were left untransfected or transfected with a mixture of lipofectamine2000 and a control SiRNA (SiCtrl) and the expression of MxA protein was assessed 24 hours after transfection. As a positive control, HAEC were treated with 100U/ml IFNβ. **(B)** HAEC were either left untransfected (Unt.) or transfected with a mixture of Hiperfect and SiCtrl and the expression of MxA protein was assessed 24 hours after transfection. As a positive control, HAEC were treated with 100U/ml IFNβ. β-actin was used as a loading control. One representative experiment is shown.

As expression of β1 integrins and PDGFRα was common to all four cell types, I then evaluated their involvement in entry of both TB40/E and TB40/F. hEC and pEC transfected with a SiCtrl or SiRNA specific for β1 integrins (Fig 7) or PDGFRα (Fig 8), were infected with either TB40/E (hEC and pEC) or TB40/F (only pEC), and the expression of IE proteins was assessed 24 hpi by Western blot. The consequence of the silencing of receptors on TB40/F infection of hEC could not be assessed since fibrotropic HCMV strains do not translocate to the nucleus of hEC, a prerequisite for initiation of viral replication, and therefore hEC do not express IE proteins (265). In both HAEC and HMVEC, silencing of the β1 integrin subunit resulted in a decreased expression of IE proteins compared to cells transfected with an irrelevant SiCtrl,

confirming the involvement of β1 integrins in HCMV entry into hEC (81). SiRNA being designed for human cells, silencing of β1 integrins was less efficient in pEC when compared to hEC. As a consequence, the effect of silencing on HCMV infection was not optimal. When PEDSV.15 cells were silenced for β1 integrin subunit expression, a decreased expression of IE proteins was observed in both TB40/E- and TB40/F-infected PEDSV.15 cells. Surprisingly, silencing of β1 integrins in 2A2 cells did not result in a decreased IE protein expression in both TB40/E- and TB40/F-infected 2A2 cells (Fig. 7).

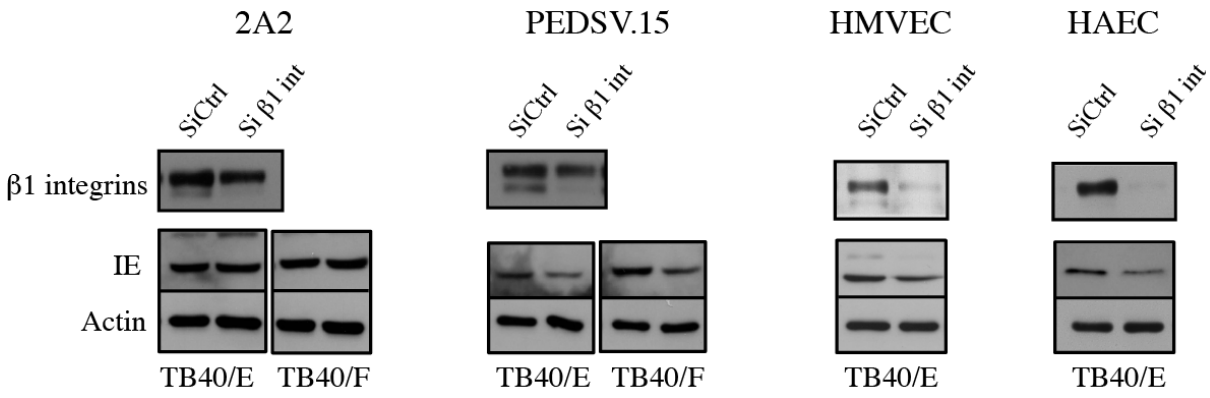

Fig. 7: Incidence of silencing of β1 integrin subunit expression on entry into EC of two strains of HCMV. hEC and pEC were transfected with either control SiRNA (SiCtrl) or β1 integrins SiRNA. Cells were infected 24h post-transfection and IE expression was assessed 24 hpi by Western Blot. β-actin was used as a loading control. One representative experiment out of three is shown.

Silencing of PDGFRα in both pEC resulted in a decreased expression of IE proteins 24 hpi in both TB40/E- and TB40/F-infected cells, although the inhibition was weaker in TB40/F- compared to TB40/E-infected PEDSV.15 cells. In contrast, silencing of PDGFRα did not modulate IE protein expression in TB40/E-infected hEC, which supports the literature demonstrating that PDGFRα might not be involved in HCMV entry into hEC (301) (Fig. 8). It is noteworthy to mention that both hEC display a weak expression of PDGFRα compared to both pEC, which would explain why silencing of PDGFRα does not affect HCMV infection of hEC.

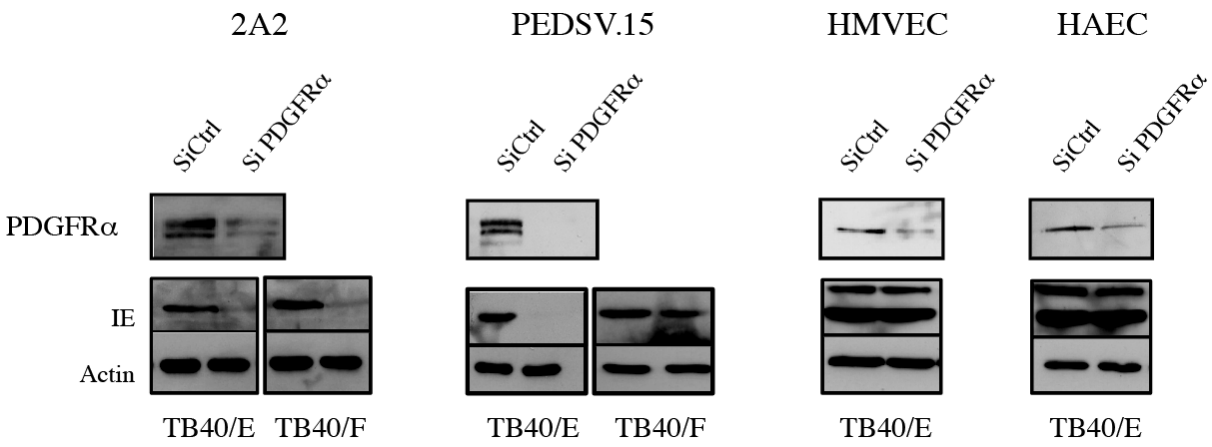

Fig. 8: Incidence of silencing of PDGFRα expression on entry into EC of two strains of HCMV. hEC and pEC were transfected with either control SiRNA (SiCtrl) or PDGFRα SiRNA. Cells were infected 24h post-transfection and IE expression was assessed 24 hpi by Western Blot. β-actin was used as a loading control. One representative experiment out of three is shown.

The assessment of the involvement of αV and β3 integrin subunits in HCMV entry into hEC and pEC was more challenging. Indeed, depending on the SiRNA used, the transfection conditions are rather unlikely to stay consistent between two different SiRNAs and as a consequence, a condition optimal for one specific SiRNA might not be ideal for another SiRNA. It was also documented that SiRNA vary markedly in their gene-silencing efficacy. These two complications rendered the silencing of αV and β3 integrin subunits very challenging. Despite several attempts, I was not able to select an effective SiRNA sequence targeting the expression of αV and β3 integrin subunits. Therefore, I was not able to confirm the involvement of αV and β3 integrin subunits in HCMV entry into hEC and to assess their role in HCMV entry into pEC.

6.2 Analysis of the mechanisms of HCMV internalization

6.2.1 Involvement of endocytosis in HCMV internalization

6.2.1.1 Incidence of specific inhibitors of endocytosis on cell proliferation, cell cycle progression and HCMV binding

In order to investigate potential HCMV entry pathways in pEC and compare them with the entry mechanisms used by HCMV to enter hEC, I used agents that are known to inhibit the various endocytic pathways. It is to be noted that some of the

agents used in this study also have other functions than inhibiting endocytosis. In my settings, I treated the cells prior to infection and the drugs were removed after 1h30 min of infection compared to the literature were drugs were left the entire duration of the experiment (8 or 20 hours) or later during infection to investigate processes subsequent to virus entry (238). In my settings I assumed that the main effect of the drugs would be linked to virus entry, therefore I referred to these drugs as inhibitors of entry. I first determined the toxicity of inhibitors of the clathrin-mediated endocytosis (chlorpromazine), the lipid rafts (filipin), the dynamin 2-dependent pathways (dynasore) and macropinocytosis (EIPA) by a proliferation assay (AlamarBlue® assay) and a cell cycle arrest assay (Fig. 9 and 10, respectively). For the proliferation assay, cells were treated for 2.5 hours, washed and incubated for another 24h in fresh medium. The next day proliferation was assessed with an AlamarBlue® assay. The drug treatment did not affect cell proliferation, represented by a similar proliferation capacity of treated cells compared to untreated cells (Fig. 9). For the cell cycle arrest assay, cells were treated as for the inhibition assay described in chapters 6.2.1.2 and 6.2.1.3: briefly, cells were treated for 2.5 hours with the different inhibitors, then washed, and incubated for another 6.5 hours in fresh medium. Cells were then fixed, stained with propidium iodide and analysed by flow cytometry. As a positive control, cells were treated with nocodazole during the duration of the experiment (9 hours). Nocodazole is an agent that binds free tubulin subunits, preventing the incorporation of tubulin in microtubules and therefore preventing microtubules polymerization, which induces a stop in the G2/M phase. The percentage of cells in each cell cycle phase varied from one cell type to another, however no significant differences in the cell repartition in each phase was observed between untreated and treated cells, indicating that the doses used in this study do not stop the cell cycle progression. As expected, when cells were treated with nocodazole, most of the cell population was blocked in the G2/M phase. As an example, in 2A2 cells, 77.4% of cells were in the G2/M phase when treated with nocodazole as compared to 15.8% for untreated cells (Fig. 10). To rule out that the absence of effect of the different inhibitors observed at 8h post treatment was due to an insufficient time for the cells to proliferate, the same experiment was carried out with a longer incubation time in fresh medium. Therefore, in a second experiment, cells were incubated for 24h after treatment and the repartition in the cell cycle was analysed.

After 24h, no modulation was observed confirming the non-toxicity of the inhibitors (data not shown).

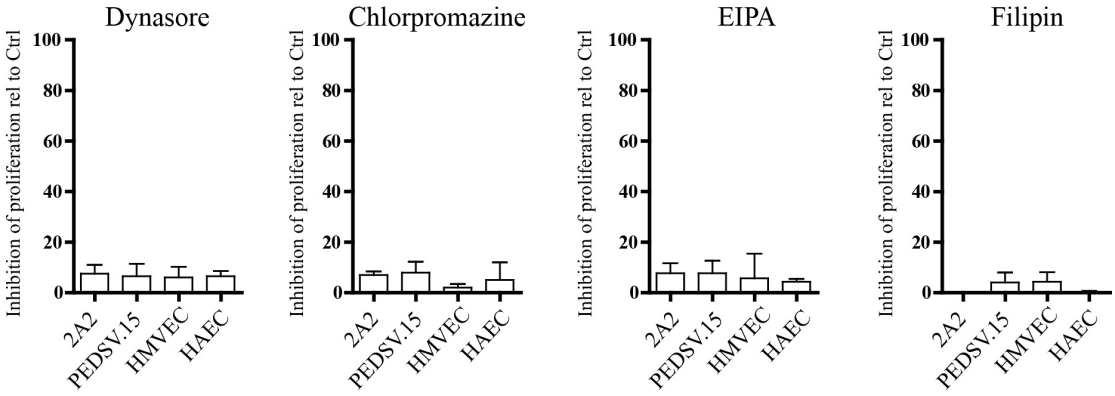

Fig. 9: Analysis of the effect of the different inhibitors of endocytosis on cell proliferation. hEC and pEC were treated with the different inhibitors and the proliferation was assessed by an AlamarBlue® assay.

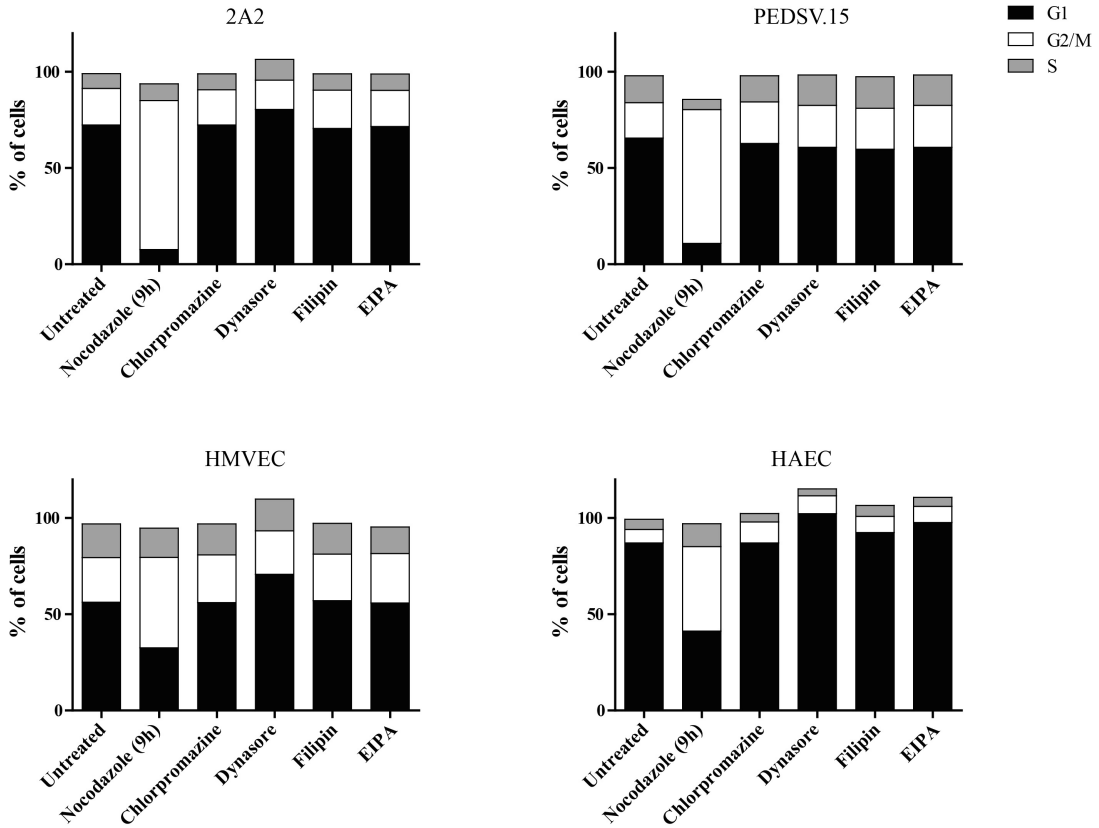

Fig. 10: Incidence of the inhibitors of endocytosis on cell cycle progression. hEC and pEC were treated with the different inhibitors for 2.5 hours and the cell cycle progression was assessed 8h later by flow cytometry.

An additional study was undertaken to investigate whether the inhibitors of endocytosis used in this study affect virus binding. Indeed, a potential effect of these drugs could result in altering virus binding by downregulation of the expression of receptors (chlorpromazine has been shown to downregulate receptor recycling (306)). Cells pre-treated with the different inhibitors were infected with TB40/F and the accumulation of the tegument protein pp65 was analysed by Western blot. Pre-treatment of hEC and pEC with chlorpromazine (C), dynasore (D), filipin (F) and EIPA (E) did not modulate the accumulation of pp65 when compared to untreated cells (U). As a control for inhibition of binding capacities of viral particles, TB40/F was pre-incubated with heparin (100µg/ml) and as expected, almost no pp65 was detected (Fig 11). It is noteworthy to mention that binding was only assessed for TB40/F and not for TB40/E because almost no pp65 is detected in TB40/E-infected EC (discussed in chapter 6.3.2); however it is known that the tropism of the virus does not modulate binding capacities of HCMV (265) and as a consequence, I assumed that the binding capacity of both strains was similarly modulated by the different drug treatments.

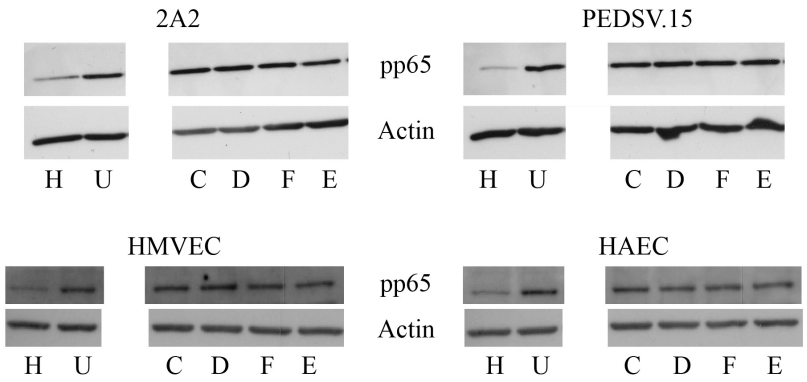

Fig. 11: Effect of inhibitors of endocytic pathways on HCMV binding. hEC and pEC were pre-treated with different inhibitors and infected for 1h at 4°C to allow binding and the viral tegument protein pp65 was detected by Western blot. As a control, HCMV was preincubated with heparin (100µg/ml). β-actin was used as a loading control. H: heparin, U: untreated, C: chlorpromazine, D: dynasore, F: filipin and E: EIPA.

6.2.1.2 Implication of endocytic pathways and lipid rafts in entry into hEC and pEC of the endotheliotropic strain TB40/E

A number of different internalization and trafficking pathways are utilized by animal viruses to enter host cells. The best documented ones include direct fusion at the membrane, endocytosis involving lipid rafts, clathrin-mediated endocytosis and

uptake via caveolae (both dependent on the activity of a large GTPase, dynamin-2, for the release of endocytic vesicle from the plasma membrane (63, 120, 208)), macropinocytosis and phagocytosis (182, 258). Caveolae-mediated endocytosis transports very small cargos since the diameter of caveolin-coated vesicles can only reach up to 100 nm. The HCMV diameter being around 200 to 300 nm, I reasoned that regarding the size of the vesicles and viral particles, caveolae-mediated endocytosis might not likely be involved in HCMV entry into EC.

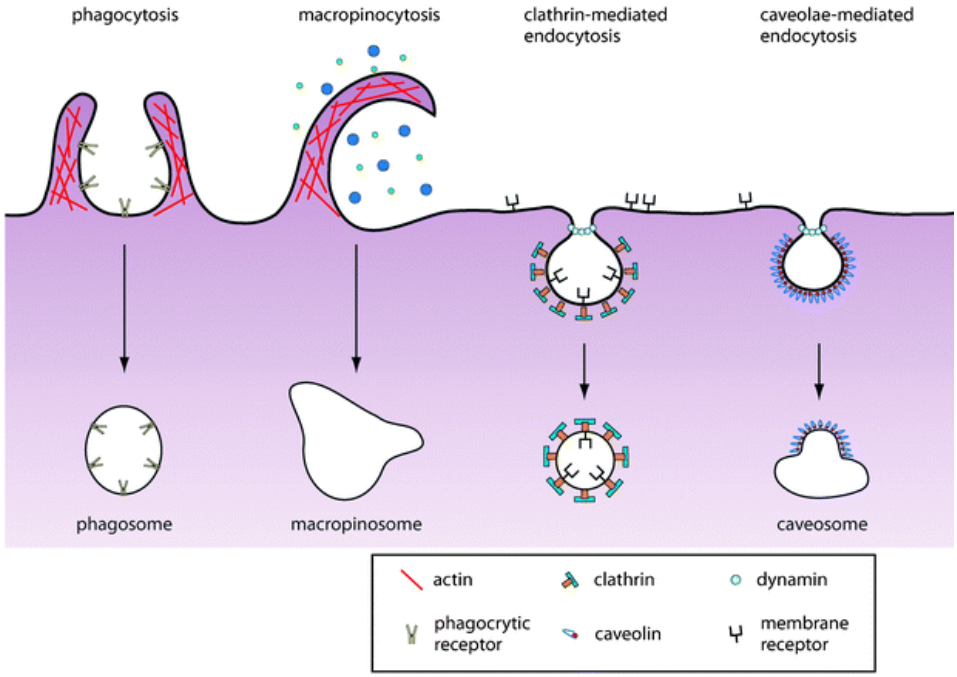

Fig. 12: Scheme illustrating the different endocytic pathways involved in virus entry (adapted from Chou et al. (45)).

HAEC, HMVEC, PEDSV.15 and 2A2 cells pre-treated with nontoxic dose of inhibitors determined in the previous chapter were infected at different MOI in order to have the same percentage of IE expressing cells: an MOI of 1 for HAEC, HMVEC and PEDSV.15 cells and an MOI of 0.5 for 2A2 cells since these cells are more sensitive to infection (185). Entry was then assessed by the expression of IE proteins by immunofluorescence 8 hpi (Fig. 13A). When EC were treated with dynasore, a molecule interfering *in vitro* with the GTPase activity of dynamin 2 (175), the expression of IE proteins was inhibited by about 80% for all four cell types, suggesting that, similarly to hEC, TB40/E entry into pEC is dynamin 2 dependent. Because I observed a strong inhibition with all the four cells types, as a control for specificity of dynasore, the same experiment was conducted with human fibroblasts

(MRC-5) in which HCMV enters via direct fusion of the viral envelope with the plasma membrane (54), thus dynasore is not expected to have any effect on infection. As expected, no inhibition of the expression of IE proteins in dynasore-treated compared to untreated MRC-5 was observed (Fig 13B). To assess the involvement of clathrin-mediated endocytosis, the specific inhibitors chlorpromazine, a cationic amphiphilic drug that causes clathrin to localize and accumulate in late endosomes (306), was used. When EC were treated with chlorpromazine, a moderate inhibition of about 20% for 2A2 cells, 30% for HMVEC and 40% for PEDSV.15 cells and HAEC was observed. When cells were treated with the inhibitor of macropinocytosis EIPA, an inhibitor of Na(+)/H(+) exchanger, an inhibition of 30% was observed for 2A2 cells and at least 40% for HAEC, HMVEC and 2A2 cells (Fig. 13A). These findings demonstrate that similar to entry into hEC, TB40/E enters pEC by a dynamin-2 dependent mechanism and macropinocytosis. However, despite taking care to remove all drugs we cannot completely exclude some remaining effects which could affect post-entry, which are irreversible despite removal. One direct assay to assess the effect of the drugs on entry would be to quantify the viral genome delivered in the cytoplasm.

Lipid rafts, also referred to as microdomains, are highly dynamic structures of the plasma membrane that contain high concentration of cholesterol and glycosphingolipids. Membrane proteins involved in cell signalling have been observed in these microdomains, leading to the consent that lipid rafts play a role in signalling transduction (reviewed in (261)). These membranes proteins include amongst others EGFR and PDGFR (169, 188). Regarding HCMV infection, Wang et al. demonstrated that lipid rafts have an important role in regulating the interaction between EGFR and αVβ3 integrin and its signalling pathway, leading then to virus internalization. One approach for studying the function of lipid rafts involves depleting cells of cholesterol. Interactions between cholesterol and sphingolipids are important for holding lipid raft structures together, therefore the integrity of these microdomains can be disrupted by treating cells with certain agents such as filipin which binds to and sequester cholesterol. I assessed whether the removal of cholesterol from the plasma membrane and indirectly the disruption of lipid rafts impacts TB40/E entry into pEC. After treating the cells with filipin, I observed an inhibition of IE expression between 40 and 45% and 30% for hEC and pEC,

respectively, suggesting an involvement of lipid rafts in TB40/E entry into EC (Fig. 13C).

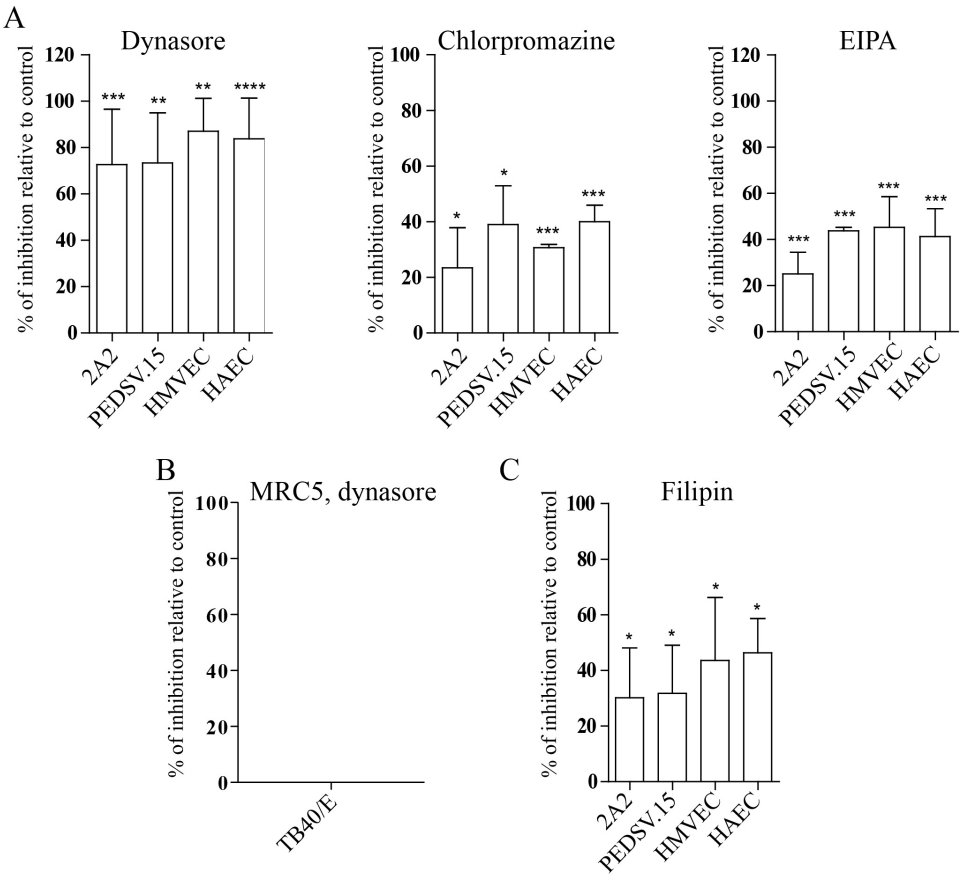

Fig. 13: Comparison of the incidence of the treatment of inhibitors of endocytosis on the endotheliotropic strain TB40/E entry into hEC and pEC. EC were pretreated for 1h with various drugs inhibiting **(A)** endocytosis and phagocytosis (dynasore), clathrin-mediated endocytosis (chlorpromazine) or macropinocytosis (EIPA) or **(C)** lipid rafts (filipin) and were inoculated with TB40/E. **(B)** Human fibroblasts (MRC-5) were pretreated for 1h with dynasore and infected with TB40/E. Infection was assessed by detection of IE proteins at 8hpi. *: <0.05, **: <0.001, ***: <0.003, ****: <0.001. One representative experiment is shown for MRC-5.

6.2.1.3 Implication of endocytic pathways and lipid rafts in entry into hEC and pEC of the fibrotropic strain TB40/F

Sinzger et al. demonstrated that contrary to endotheliotropic HCMV strains (e.g. TB40/E), fibrotropic strains do not translocate to the nucleus of hEC and consequently cannot initiate viral replication (265). The expression of IE proteins being the main readout, I was able to assess the implication of endocytic pathways in TB40/F-infected pEC. When pEC were treated with dynasore, an inhibition >80% was observed for both cell types suggesting that TB40/F entry into pEC is dynamin 2

dependent (Fig. 14A). As previously, the pre-treatment of MRC-5 with dynasore did not inhibit IE expression (Fig. 14B). When pEC were treated with chlorpromazine, no effect on IE protein expression was observed, suggesting that TF40/F entry does not occur through a clathrin-dependent pathway. When pEC were treated with EIPA, IE protein expression was inhibited by less than 20% for both 2A2 cells and a non-significant inhibition of the expression of IE proteins was observed for PEDSV.15 cells, suggesting that macropinocytosis is involved to a lesser extent in TB40/F entry into pEC (Fig. 14A). When cells were treated with filipin, no significant inhibition was observed suggesting that entry of TB40/F into pEC does not require lipid rafts (Fig. 14C). These results suggest that to enter pEC, TB40/F uses mainly a dynamin-2 dependent mechanism and lipid rafts are not involved.

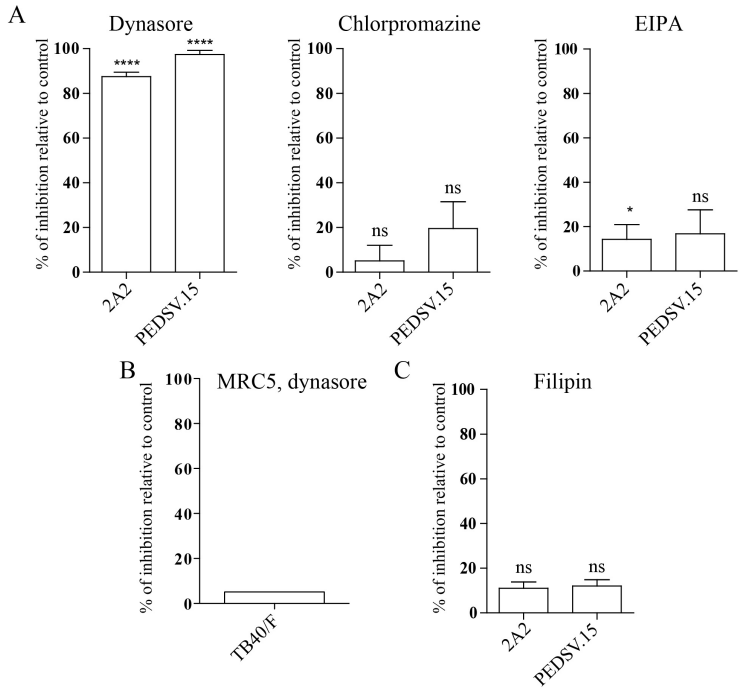

Fig. 14: Comparison of the incidence of the treatment of inhibitors of endocytosis on the fibrotropic strain TB40/F entry into hEC and pEC. pEC were pre-treated for 1h with various drugs inhibiting **(A)** endocytosis and phagocytosis (dynasore), clathrin-mediated endocytosis (chlorpromazine) or macropinocytosis (EIPA) or **(C)** lipid rafts (filipin) and were inoculated with TB40/F. **(B)** Human fibroblasts (MRC-5) were pretreated for 1h with dynasore and infected with TB40/F. Infection was assessed by detection of IE proteins at 8hpi. *: 0.05, ****: <0.001. One representative experiment is shown for MRC-5.

6.2.2 Involvement of the endosomal pH acidification in entry of both HCMV strains

Immediately following internalization by endocytosis, the acidification of endocytic vesicles triggers a conformational change in the viral glycoproteins and the release of the viral capsid into the cytoplasm. Some viruses also enter cells in a pH-independent manner via direct fusion of their envelope with the plasma membrane. Depending on the cell type, HCMV has the capability to use these two distinct mechanisms. I thus evaluated the importance of the endosomal pH acidification in the release of HCMV capsids in the cytoplasm by treating the cells with an inhibitor of the vacuolar H^+-ATPase (bafilomycin A1, BafA1) (321). First, as for the inhibitors of the different endocytic pathways, the toxicity of BafA1 on cell proliferation and cell cycle progression was evaluated. As for the previous inhibitors, BafA1 did not inhibit proliferation neither it blocked the cell cycle progression (Fig 15A and B). I then evaluated whether EC pre-treatment with BafA1 prevented HCMV binding, and as previously, the drug did not have any effect on virus binding (data not shown). The next step was to ensure that the concentration of BafA1 used in the experiment inhibited the acidification of the intracellular acidic vesicles. For this purpose, EC were stained with acridine orange following treatment with or without BafA1 and visualized under a fluorescence microscope. The emission spectrum of acridine orange (AO) depends on the local concentration of the compound. AO is green in the nucleus whereas in acidic compartments it gets protonated and sequestered. In these low pH conditions, AO will emit orange light when excited with blue light. Figure 15C shows EC that were incubated with acridine orange. The figure shows that the red fluorescence representing the acidic vesicles disappeared when EC were treated with 20 nM of BafA1. This result confirms that 20 nM of BafA1 are enough to inhibit endosomal acidification. To assess whether endosomal pH acidification is required in the release of HCMV capsids in the cytoplasm, hEC and pEC pretreated with BafA1 were infected with TB40/E (pEC and hEC) or TB40/F (pEC only) and IE expression was evaluated 8 hpi by immunofluorescence. In both TB40/E-infected pEC, pre-treatment with BafA1 resulted in an inhibition of IE expression of 30 and 50% for 2A2 cells and PEDSV.15 cells, respectively. Similar results were observed with hEC, suggesting that TB40/E entry into hEC and pEC involved endosomal pH acidification (Fig. 15D). An inhibition of 30% was observed in TB40/F-infected PEDSV.15 cells

suggesting a relative involvement of the pH in TB40/F entry into these cells. Surprisingly, pretreatment of 2A2 cells with BafA1 resulted in an inhibition of only 15%, suggesting that in these cells, pH acidification is dispensable for TB40/F entry into 2A2 cells.

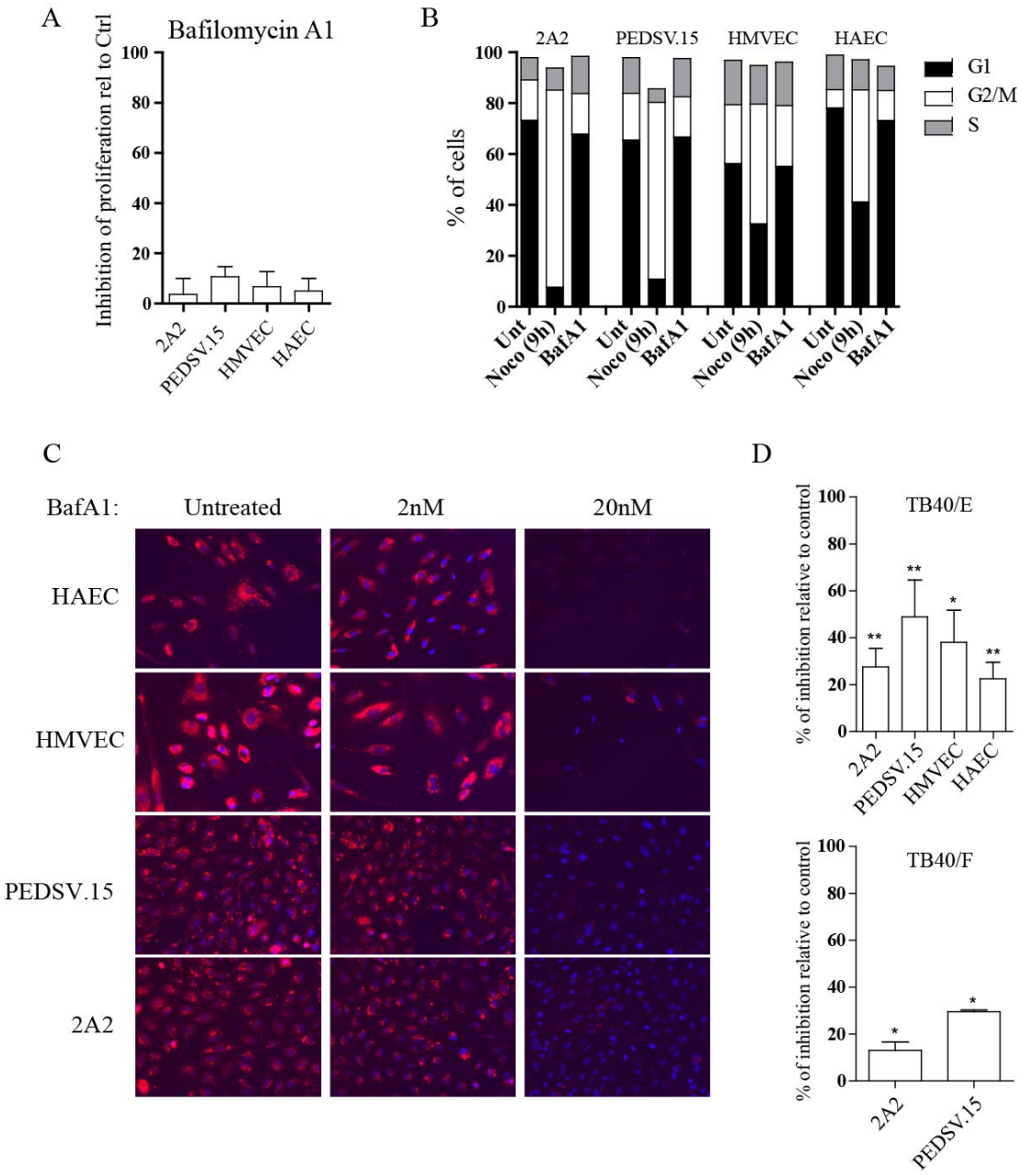

Fig. 15: Comparison of the involvement of the endosomal pH acidification in the entry into hEC and pEC of the endotheliotropic TB40/E and the fibrotropic TB40/F strains. hEC and pEC were treated with bafilomycin A1 (BafA1) and **(A)** the proliferation or **(B)** the cell cycle progression were assessed. Noco (9h): Nocodazole treated cells for 9h. **(C)** hEC and pEC treated or not with BafA1 (2 or 20nM) for 1 hour were incubated in medium containing 5 µg/ml of acridine orange for 10 min. The acidic compartments are presented as a red staining in the untreated EC. **(D)** Cells were pretreated for 1h with a drug inhibiting endosomal pH acidification (Bafilomycin A1) and were inoculated with TB40/E or TB40/F. Infection was assessed by detection of IE at 8hpi. *: <0.05, **: <0.001. **(C)** One representative experiment out of two is shown.

6.2.3 Involvement of the actin and microtubule cytoskeleton in HCMV internalization and translocation

It has been reported that HCMV entry into epithelial and endothelial cells is actin dependent (19). Entry and trafficking of herpesvirus in general was shown to be dependent on both the actin and the microtubule cytoskeleton, which cooperate in order to deliver the viral genome to the nucleus of infected cells (173). I thus assessed whether HCMV entry into pEC also requires an intact actin (Fig. 16) and microtubule (Fig. 17) cytoskeleton. I used cytochalasin D, a pharmacological inhibitor that binds to actin barbed ends, which consequently blocks polymerization and depolymerisation at the plus end. I analysed the effects of this inhibitor on HCMV infection. First, the toxicity and the effect of this inhibitor on HCMV binding were assessed. Cytochalasin D did not inhibit cell cycle progression and no inhibition of binding was observed (data not shown). Besides, we evaluated whether the concentration used was equally active in hEC and pEC. To do so, I stained the pre-treated cells with phalloidin (a toxin know to bind fibrous actin) and looked at the structure of the actin cytoskeleton. For all four cell types, the cytochalasin D pre-treatment resulted in a disassembly of the actin cytoskeleton represented by the absence of a fibrous actin staining (data not shown). HAEC, HMVEC, PEDSV.15 cells and 2A2 cells pre-treated with cytochalasin D were infected with TB40/E (Fig. 16A) or TB40/F (Fig. 16B), and entry was assessed by the expression of IE proteins by immunofluorescence 8 hpi. When pre-treated with cytochalasin D, infection with TB40/E of HAEC and HMVEC was completely inhibited as assessed by a nearly 100% reduction of IE protein expression, confirming that actin remodelling is essential for HCMV entry into EC. After pre-treatment of 2A2 cells and PEDSV.15 cells, an inhibition of 70% and only 35% was observed, respectively, this suggests that an intact actin cytoskeleton is required for entry of TB40/E into 2A2 cells but dispensable for entry into PEDSV.15 cells (Fig. 16A). In contrast, when pre-treated 2A2 cells and PEDSV.15 cells were infected with TB40/F, no inhibition of IE expression was observed, suggesting that in these conditions, TB40/F enter pEC in an actin-independent manner (Fig. 16B). Because of the weaker effect of cytochalasin D on the inhibition of pEC infection with HCMV, we analysed the consequences of cytochalasin D treatment on fibrous actin by phalloidin staining. After treatment with cytochalasin D, we observed a conversion of F-actin from long fibers in untreated 2A2 to punctate structures in

treated 2A2 (Fig. 16C), confirming the effectiveness of the cytochalasin D in 2A2. Similar results were obtained with PEDSV.15 (data not shown).

To investigate the involvement of the microtubule cytoskeleton in HCMV entry / nuclear translocation, I used nocodazole, which prevents microtubule polymerization. As for cytochalasin D, the toxicity and the effect on HCMV binding capacity after treatment with nocodazole was assessed. For the cell cycle arrest assay, EC were treated with nocodazole for 2h30 (1 hour pre-treatment followed by a 1h30 treatment with the virus) (in contrast to the positive control where a lower dose of nocodazole is incubated for the duration of the entire experiment, i.e. 9h) then washed and incubated with fresh medium for 6h30. The 2h30 treatment did not inhibit proliferation or stop the cell cycle progression neither did it prevent virus binding (data not shown). I then assessed the impact of such treatment on HCMV infection. HAEC, HMVEC, PEDSV.15 cells and 2A2 cells pre-treated with nocodazole were infected with TB40/E (Fig. 17A) or TB40/F (Fig. 17B), processed and analysed as previously described. When HAEC and HMVEC were pre-treated with nocodazole, an inhibition of IE expression of 90% and 65% was observed, respectively, confirming the involvement of microtubules in the nuclear translocation of viral capsid. An inhibition of 70% was observed in treated PEDSV.15 cells, suggesting as well an involvement of microtubules in TB40/E nuclear translocation. In contrast, a weak inhibition of ~20% was observed in 2A2 cells, suggesting that in these cells, an intact microtubule cytoskeleton might not be necessary for trafficking (Fig. 17B). An inhibition of 30% was observed in TB40/F-infected PEDSV.15 cells suggesting a partial involvement of microtubules in translocation. In contrast, an inhibition <10% was observed for 2A2 cells, suggesting that microtubules are not involved in trafficking of TB40/F particles in 2A2 cells. As previously, we confirmed the effectiveness of nocodazole treatment of pEC on microtubule organization by a tubulin staining. In untreated 2A2, tubulin staining showed a normal tubular microtubule organization. In contrast, in nocodazole treated 2A2, the tubular structure was absent (Fig. 17C), confirming the effectiveness of nocodazole in 2A2. Similar results were obtained with PEDSV.15 (data not shown).

These results suggest that the involvement of the actin and the microtubule cytoskeleton depends on both viral tropism and the vascular bed origin of the cells.

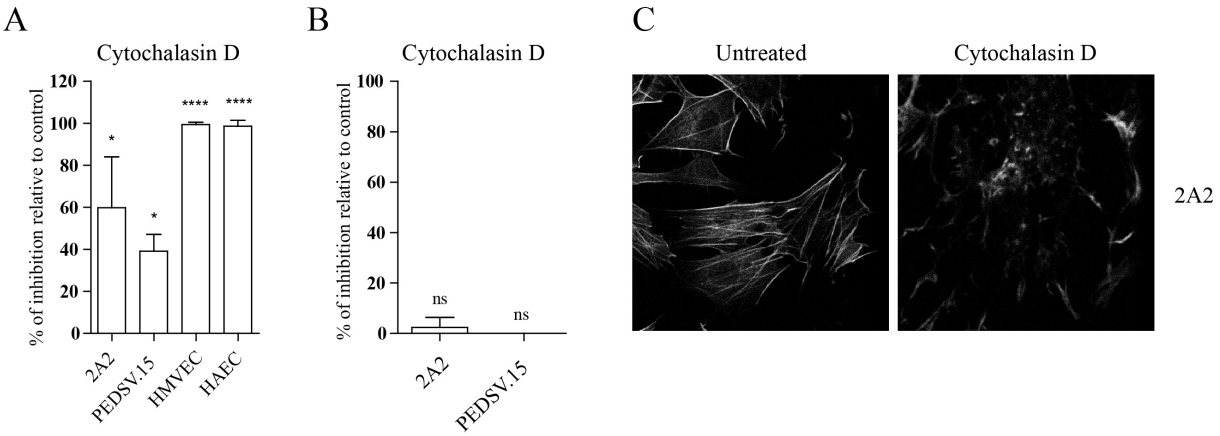

Fig. 16: Involvement of the actin cytoskeleton in entry into hEC and pEC of the endotheliotropic TB40/E and the fibrotropic TB40/F HCMV strains. hEC and pEC were pretreated for 1 hour with a specific inhibitor of actin polymerization before being inoculated with **(A)** TB40/E or **(B)** TB40/F. Results are expressed as the percentage of inhibition of infection relative to untreated cells, and bars represent mean values ± SD of three independent experiments. Comparisons were performed using t test and significant differences are indicated as follow: *: < 0.05, ****: <0.0001. The mean of three independent experiments is shown. **(C)** Effect of cytochalasin D on fibrous actin organization. 2A2 were exposed to cytochalasin D for 1 hour, fixed and stained with rhodamine–phalloidin.

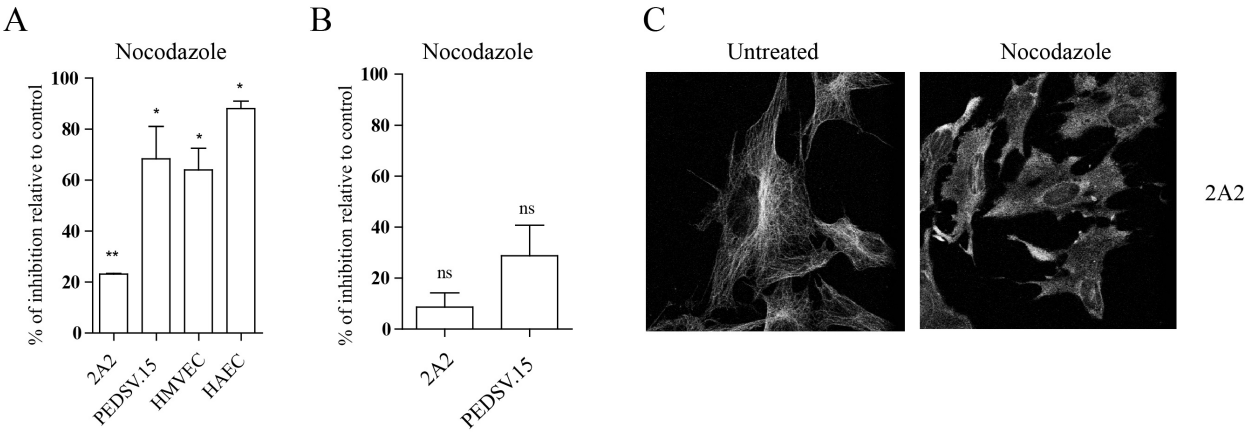

Fig. 17: Involvement of the microtubule cytoskeleton in entry into hEC and pEC of the endotheliotropic TB40/E and the fibrotropic TB40/F HCMV strains. hEC and pEC were pretreated for 1 hour with a specific inhibitor of microtubule polymerization before being inoculated with **(A)** TB40/E or **(B)** TB40/F. Results are expressed as the percentage of inhibition of infection relative to untreated cells, and bars represent mean values ± SD of three independent experiments. Comparisons were performed using t test and significant differences are indicated as follow: *: < 0.05, **: <0.01. The mean of three independent experiments is shown. **(C)** Effect of nocodazole on microtubules organization. 2A2 were exposed to nocodazole for 1 hour, fixed and stained for tubulin.

6.3 Involvement of the host species in HCMV translocation and initiation of replication

6.3.1 Assessment of the role of the classical endolysosomal pathway in HCMV intracellular trafficking to the nucleus

Subsequent to internalization, viral particles are transported from the cell surface to the nucleus where the viral genome is released, allowing initiation of viral replication. The classical endolysosomal pathway is responsible for transport of cargo in endocytic vesicles in the cytoplasm. Viral particles contained in endocytic vesicles are delivered to early endosomes containing the early endosome antigen 1 (EEA1) (194) and are subsequently transferred to late endosomes and lysosomes expressing lysosome-associated membrane protein 2 (LAMP2) (39). Thus, I investigated whether TB40/E (Fig. 18) and TB40/F (Fig.19), once internalized in endocytic vesicles, use the classical endolysosomal pathway to traffic further into the cytoplasm. To assess this, I examined by confocal microscopy the co-localization of the HCMV viral glycoprotein B (gB) with the early endosomal marker EEA1 and the late endosomal marker LAMP2 at different intervals after infection (10, 30, 60 and 90 mpi). I reasoned that since both viral strains used endocytic pathways to enter hEC and pEC, gB will still be on the viral envelope and therefore still associated with viral particles in endocytic vesicles. Co-localization is expressed as Pearson's coefficient (which correlates the pixel intensity from the different fluorochromes) represented by values comprised between -1 and 1 and which can be interpreted as follows: the higher the coefficient is, the more two markers co-localize. After infection of hEC with TB40/E, co-localization of gB with EEA1 increased with time to reach a peak at 60 mpi followed then by a diminution of Pearson's coefficient reflecting a decreasing co-localization. Similar results were observed for pEC, however the peak was reached earlier than in hEC (30 mpi). This observation suggests that transport and / or internalization occurs faster in pEC compared to hEC (Fig. 18A). In Figure 18B, a representative image for co-localization between gB and EEA1 (green) is shown and is characterized in the merged image as a yellow staining. A similar scheme was observed for the co-localization of gB with LAMP2 in 2A2 cells, HAEC and HMVEC (Fig 18C). In PEDSV.15 cells, no LAMP2 was detected, suggesting an absence of the protein, which prevented the co-localization analysis. Figure 18D

illustrates a representative image for co-localization of gB and LAMP2 in TB40/E-infected 2A2 cells.

After infection of hEC with TB40/F, co-localization with EEA1 increased continuously until 90 mpi. In contrast, in pEC the Pearson's coefficient increased and reached a peak at 30 mpi and then decreased (Fig. 19A). No modulation of co-localization was observed between gB and LAMP2 in hEC, suggesting that TB40/F does not localize in late endosomes. In 2A2 cells, co-localization of gB with LAMP2 reached a peak at 30 mpi and then decreased (Fig. 19C). Figures 19 B and D show representative images for co-localization of gB with EEA1 and LAMP2 (both green) in TB40/F-infected 2A2 cells, respectively.

These results suggest that both strains use the classical endolysosomal pathway to traffic further into the cytoplasm of infected EC and TB40/E trafficking occurs faster in pEC compared to hEC. These results also suggest that in hEC, TB40/F might be sequestered in early endosomes, which is in accordance with the literature showing that viral particles of fibrotropic HCMV strains do not translocate to the nucleus of infected hEC. This result would then suggest that the transfer from early endosomes to late endosomes is the limiting step for fibrotropic HCMV strains nuclear translocation in hEC.

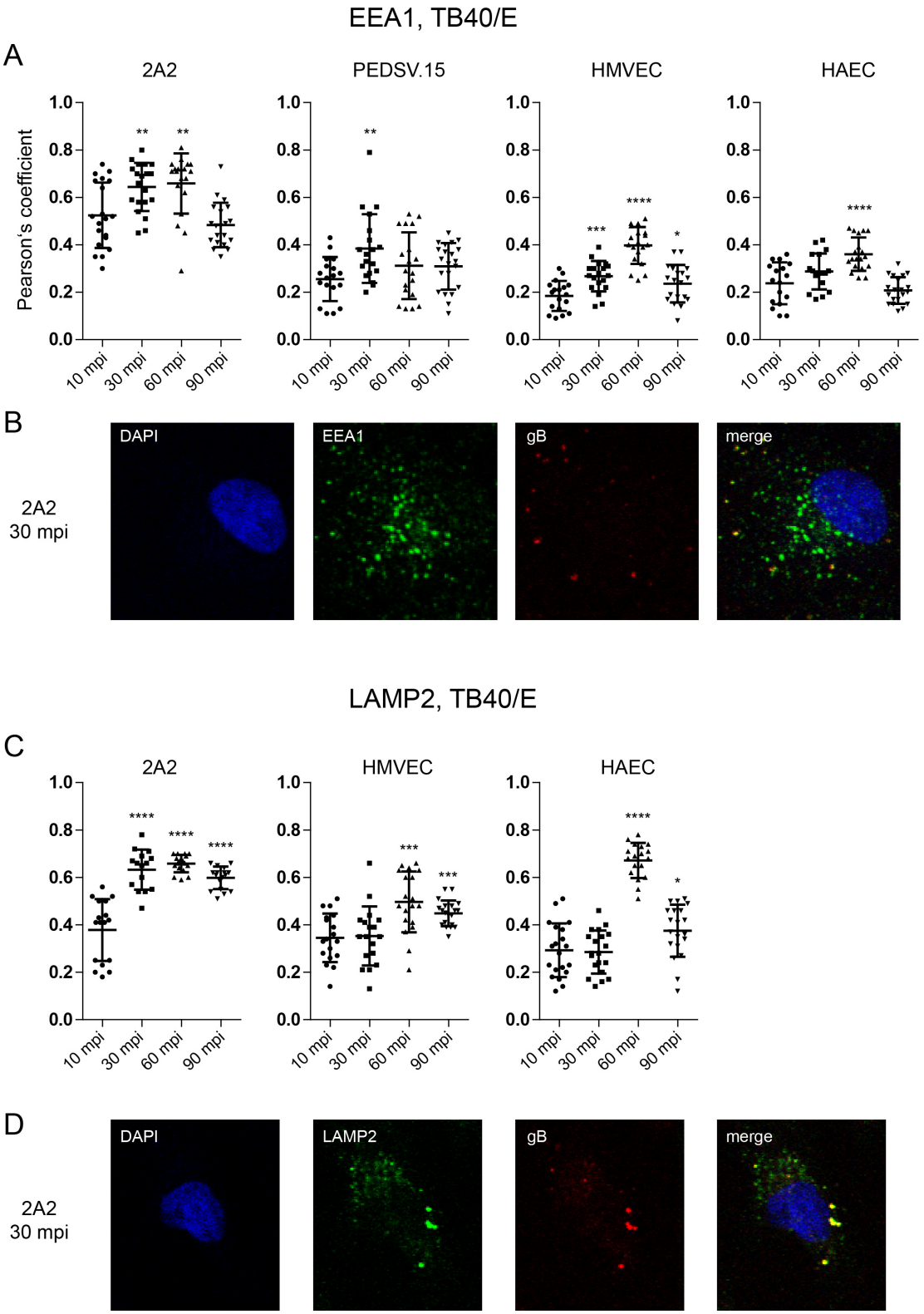

Fig. 18: Co-localization between TB40/E gB and components of the classical endolysosomal pathway. hEC and pEC were infected with TB40/E for different periods of time. **(A, C)** Co-localization analysis between gB and **(A)** EEA1 or **(C)** LAMP2 and expressed as Pearson's coefficient which correlates pixel intensity. **(B, D)** The pictures show a representative staining of **(B)** EEA1 or **(D)** LAMP2 with gB in 2A2 cells. For **(A)** and **(C)**, one representative experiment out of three independent experiments is shown, bars represent

mean values ± SD and comparisons were performed using t test and significant differences are indicated as follow: *: < 0.05, **: <0.01, ***: <0.001, ****: <0.0001.

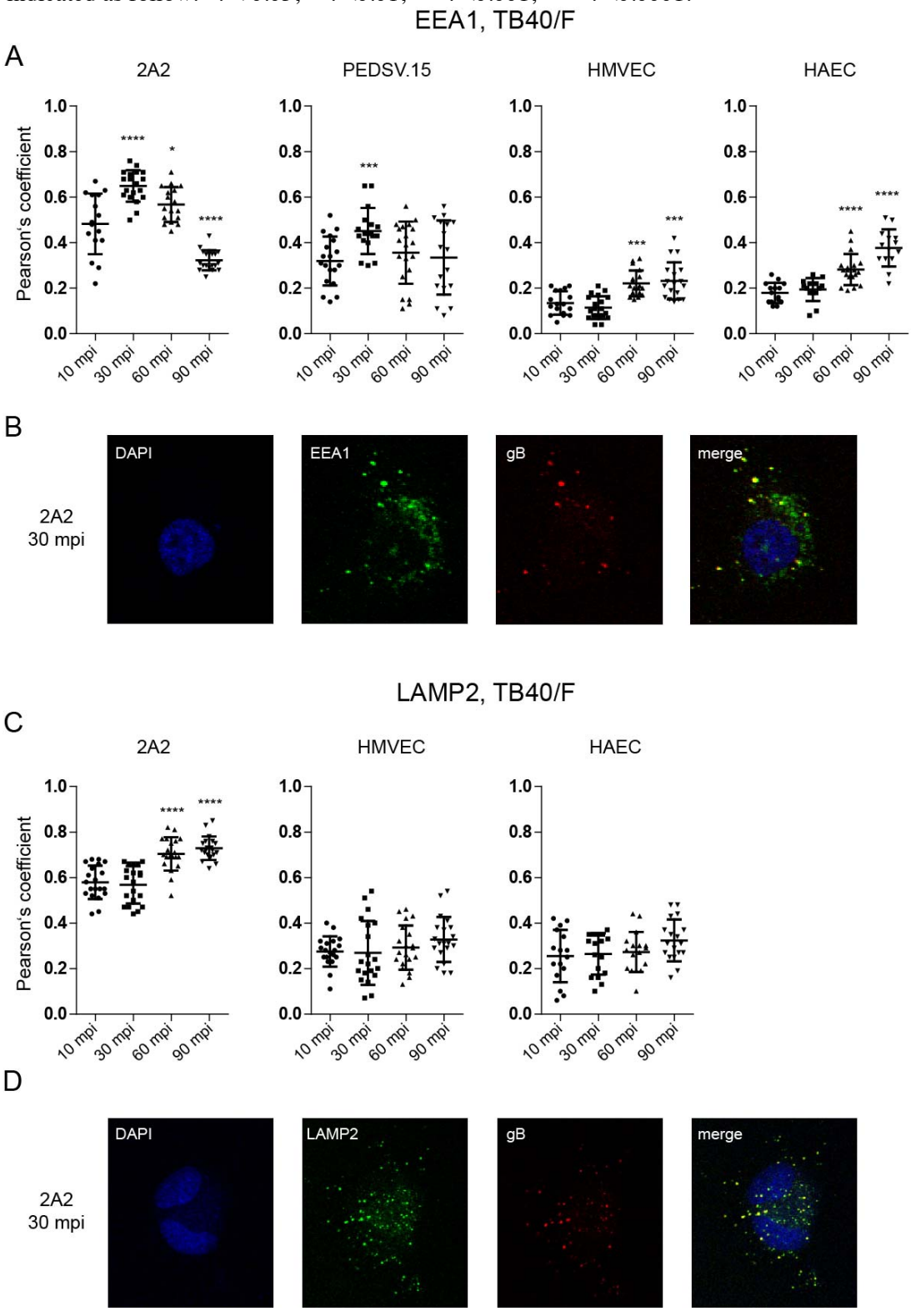

Fig. 19: Co-localization between TB40/F gB and components of the classical endolysosomal pathway. hEC and pEC were infected with TB40/F for different periods of time. **(A, C)** Co-localization analysis between gB and **(A)** EEA1 or **(C)** LAMP2 and expressed as Pearson's coefficient which correlates pixel intensity. **(B, D)** The pictures show a representative staining of **(B)** EEA1 or **(D)** LAMP2 with gB in 2A2 cells. For **(A)** and **(C)**, one representative experiment out of three independent experiments is shown, bars represent

mean values ± SD and comparisons were performed using t test and significant differences are indicated as follow: *: <0.05, **: <0.01, ***: <0.001, ****: <0.0001.

Table 4: Table recapitulating the results of colocalisation of viral particles with markers of the endolysosomal pathway.

TB40/E

	2A2	PEDSV.15	HMVEC	HAEC
EEA1	yes	yes	yes	yes
LAMP2	yes	no staining	yes	yes

TB40/F

	2A2	PEDSV.15	HMVEC	HAEC
EEA1	yes	yes	yes	yes
LAMP2	yes	no staining	NO	NO

6.3.2 Role of host species in HCMV nuclear translocation

Nuclear translocation is the last step of entry preceding the initiation of viral replication with the expression of IE proteins. Following internalization of the viral particle and release of viral capsids in the cytoplasm, capsids translocate to the nucleus and viral tegument proteins, among them pp65, are released in the nucleus. I assessed whether nuclear translocation of HCMV occurs in a similar manner in hEC and pEC by localizing and measuring pp65 of both TB40/E and TB40/F. hEC and pEC were infected at an MOI of 1 and the nuclear localisation of pp65 was analysed at different times post-infection (1-24 hpi) by immunofluorescence. As expected, no nuclear accumulation of pp65 was observed in TB40/F-infected hEC which is in accordance with previous reports showing that TB40/F and other fibrotropic strains are adsorbed and internalized by endothelial cells but fails to translocate to the nucleus and as a consequence cannot start viral gene expression (265). In contrast to hEC, shortly after inoculation (3hpi), pp65 displayed a nuclear localisation in TB40/F-infected 2A2 cells and to a lesser extend in TB40/F-infected PEDSV.15 cells. More surprisingly, no nuclear accumulation was observed in TB40/E-infected hEC as well as pEC (Fig. 20). As a control for infection, EC were stained for IE proteins and the expression was analysed 24hpi. In TB40/E-infected EC, IE proteins were detected; demonstrating that viral capsids reached the nucleus and viral replication had indeed started.

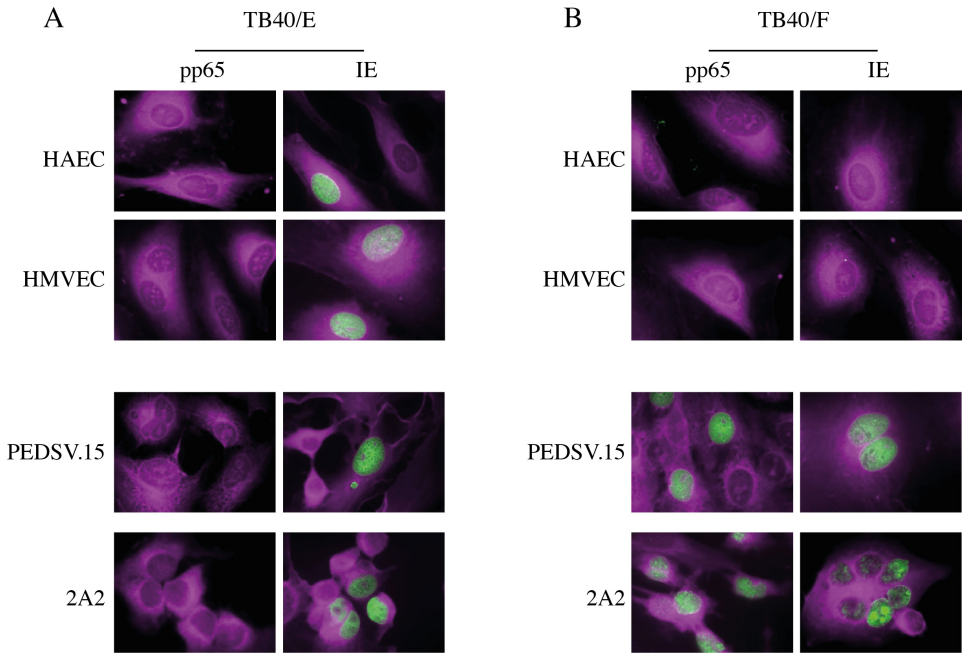

Fig. 20: Nuclear accumulation of pp65 in TB40/E- and TB40/F-infected EC. EC were infected with TB40/E **(A)** or TB40/F **(B)** at an MOI of 1 and the viral tegument protein pp65 was detected by immunofluorescence. As a control, EC were assessed for the expression of IE proteins. Pink staining: Evans Blue cytoplasmic staining, green staining: pp65 or IE as indicated. One representative experiment out of three is shown.

The absence of pp65 in the nucleus of TB40/E-infected EC could be explained by two hypotheses. The first suggestion would be a reduced expression of pp65 in TB40/E viral particles, which would result in a diminished nuclear accumulation that cannot be detected by immunofluorescence. To test this possibility, hEC and pEC were infected with TB40/E or TB40/F for different periods of times (1 to 24hpi) and the accumulation of pp65 was analysed in total extracts by Western blot. pp65 accumulation was observed in all TB40/F-infected EC and to a lesser extend in TB40/E-infected EC. In TB40/E-infected 2A2 cells and HAEC, accumulation was detected from 1 hpi to 5 hpi. In TB40/E-infected PEDSV.15 cells and HMVEC, pp65 was detected from 1 hpi to 3 hpi but the accumulation was weaker than in 2A2 cells and HAEC. In TB40/F-infected EC, the accumulation of pp65 was stronger compared to the same EC infected with TB40/E. The accumulation started at 1 hpi for all the four cell types. Depending on the cell type, the quantity of pp65 detected decreased at 8 hpi (2A2 cells and HAEC) or 5 hpi (PEDSV.15 cells and HMVEC), suggesting that the incoming pp65 might be first degraded (but is then produced later in the replication cycle, i.e. 72 hpi (data not shown)). Although the quantity of pp65 differs from one cell type to another, the pattern remains: the accumulation of pp65 is higher in TB40/F-infected EC compared to TB40/E-infected EC (Fig. 21).

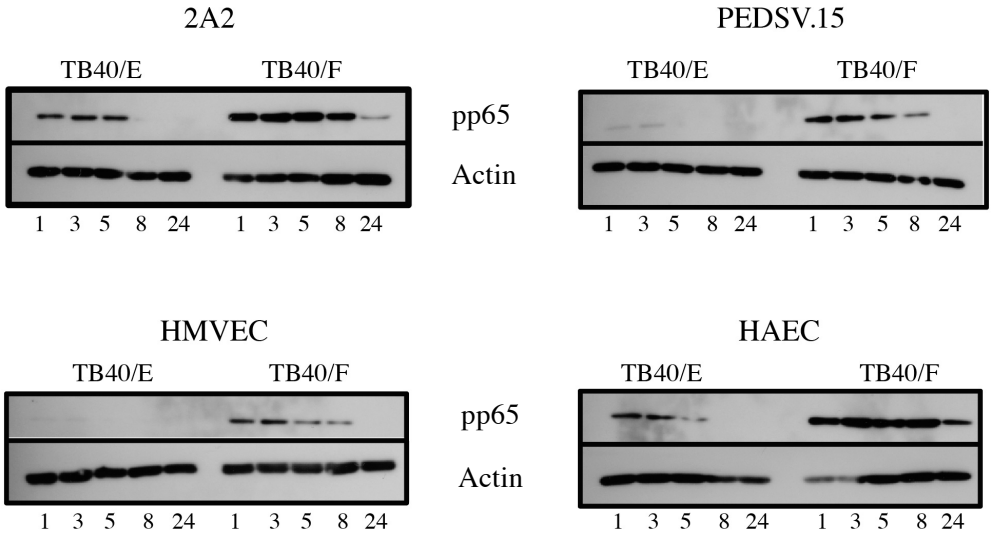

Fig. 21: Comparison of accumulation of pp65 in TB40/E- and TB40/F-infected EC. EC were infected with TB40/E or TB40/F at an MOI of 1 and the accumulation of the viral tegument protein pp65 was detected by Western blot at different times post infection (1 to 24 hpi). β-actin was used as a loading control.

The second hypothesis would be that both strains have the same amount of pp65 in the particles but more TB40/F viral particles would enter EC compared to TB40/E viral particles, which would result in higher pp65 accumulation in the cells. In accordance with this hypothesis, we previously demonstrated that, contrary to HCMV infection of hEC, pEC are more susceptible to infection with TB40/F than TB40/E (185). In order to assess whether this discrepancy was due to differential entry efficiency between the two HCMV strains, hEC and pEC were infected with either TB40/E or TB40/F at an MOI of 1, and at different times post-infection, viral DNA was quantified by real-time PCR. In order to eliminate as much bound but not internalized virus as possible, cells were extensively washed, trypsinized for 5 minutes and again extensively washed; nevertheless, we cannot exclude that some viral particles still remained attached to the EC. Internalized viral DNA increased with time and in both pEC, 2A2 cells and PEDSV.15 cells, the quantity of viral DNA was more important in TB40/F-infected cells compared to TB40/E-infected cells. Concerning hEC, similar results were observed for HAEC and HMVEC (Fig. 22). When comparing hEC and pEC, more viral DNA was detected in pEC compared to hEC. In TB40/F-infected 2A2 cells and PEDSV.15 cells, 3 and 1.5 times more viral DNA was detected compared to HMVEC and HAEC, respectively and 3 times more viral DNA was detected in TB40/E-infected pEC compared to TB40/E-infected hEC. These results suggest that i) HCMV internalization into pEC might occur faster than

into hEC and ii) more TB40/F viral particles are internalized into hEC and pEC compared to TB40/E viral particles, which could explain the higher accumulation of pp65 in TB40/F-infected compared to TB40/E-infected EC.

As a comparison, I repeated the identical experiment with the same time course in MRC-5, which are the cell type usually used for virus propagation. These cells are highly susceptible to HCMV infection, and in contrast to hEC, MRC-5 are more susceptible to fibrotropic strains compared to endotheliotropic strains. For instance, in our hands, in order to produce the same amount of virus, the inoculum of TB40/E needs to be increased by two times as compared to TB40/F. Interestingly, at 30 mpi, almost no viral DNA was detected in TB40/F-infected MRC-5 (mean of 52 HCMV DNA copies / 30ng of total DNA) compared to TB40/E-infected MRC-5 (mean of 4227 HCMV DNA copies / 30ng of total DNA). However, later during infection, as for hEC and pEC, more viral DNA was detected in TB40/F- compared to TB40/E-infected MRC-5 (Fig. 22). This result suggests that after 30 mpi, TB40/F also is internalized by fibroblasts faster than TB40/E. It is to be noted that in MRC-5, in average >10 and 30 times more viral DNA is detected in comparison to the amount of viral DNA sensed in pEC and hEC, respectively.

To be sure that the internalization discrepancy observed between these two strains was not due to a difference of the overall viral input, I quantified the amount of viral DNA in the inputs of TB40/F and TB40/E and no significant differences were observed (Fig. 22).

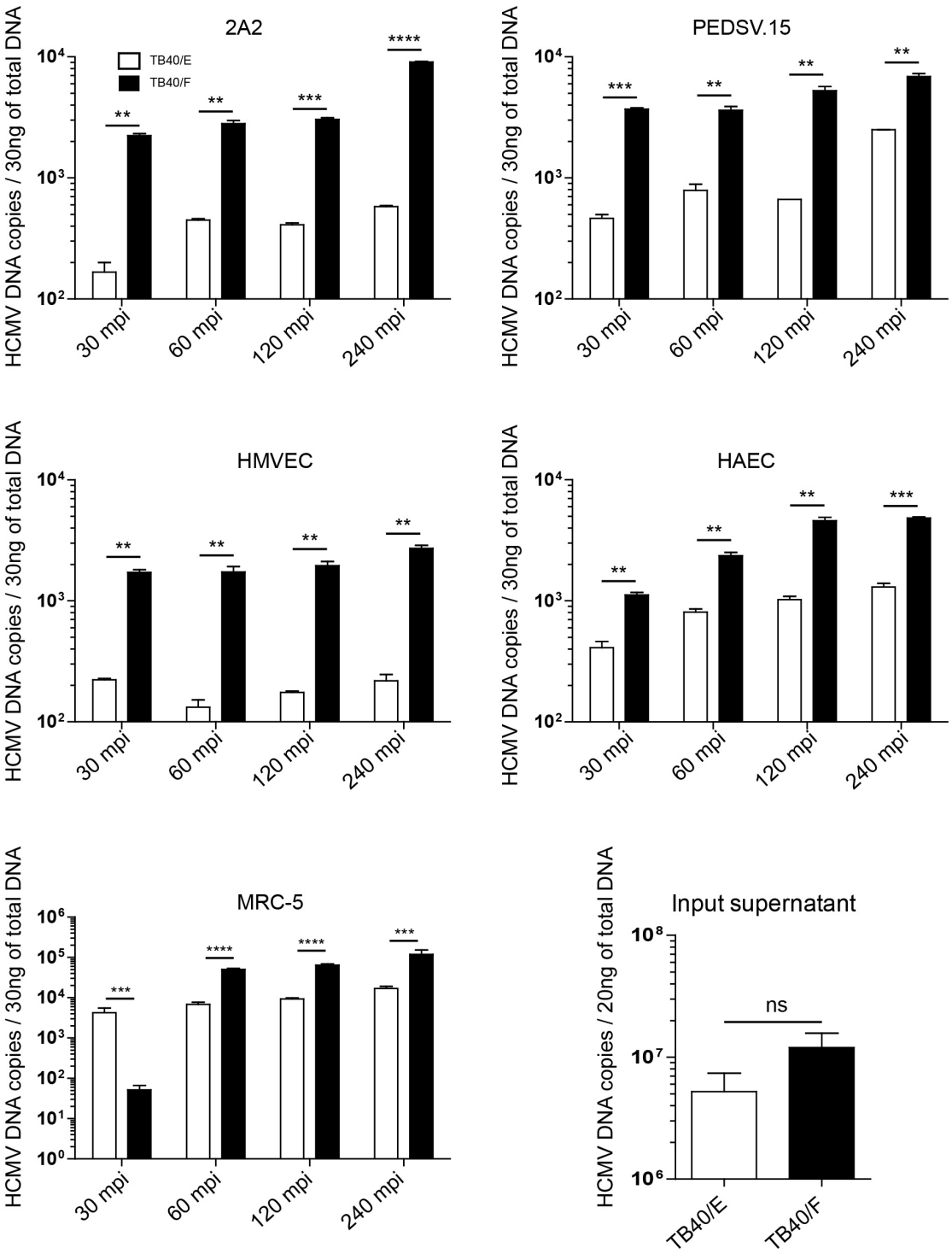

Fig. 22: Comparison of the quantity of viral DNA internalized into hEC, pEC and human fibroblasts after infection with TB40/E or TB40/F. hEC, pEC and human fibroblasts (MRC-5) were infected with either TB40/E or TB40/F (MOI of 1) for different periods of time. Total DNA was purified, amplified by real-time PCR and viral DNA was quantified. Each reaction was done in duplicate, and each point represents the average +/- standard deviation of one representative experiment out of three. For MRC-5, each reaction was done in duplicate, and the mean of two independent experiments is shown. For the input, each reaction was done in duplicate, and one representative experiment out of two is shown. White bars: TB40/E, black bar: TB40/F.

6.3.3 Effect of pp65 translocation on the initiation of HCMV replication

A previous study demonstrated that the absence of pp65 in the viral genome led to a delay in the expression of viral IE genes (291). I thus assessed whether the differences observed in pp65 nuclear accumulation could also impact the initiation of HCMV replication in pEC. Cells were inoculated with TB40/E or TB40/F and at different time post-infection (1 to 24 hpi) IE expression was assessed by Western blot. Shortly after infection (5-8 hpi), TB40/E-infected hEC and PEDSV.15 cells displayed similar amount of IE proteins, in contrast to 2A2 cells which showed a stronger expression, which is in accordance with previous results obtained in the laboratory demonstrating that 2A2 cells are more susceptible to HCMV infection (regarding IE protein expression) compared to PEDSV.15 cells, HAEC and HMVEC (185)). As expected, at 24hpi, almost no IE was detected in TB40/F-infected hEC, which is due to the incapacity of fibrotropic strains to replicate in hEC (265). In contrast, both types of TB40/F-infected pEC displayed a strong and early expression of IE proteins compared to TB40E-infected pEC (Fig. 23), suggesting that pp65 nuclear translocation is correlated to the initiation of the expression of IE proteins in pEC.

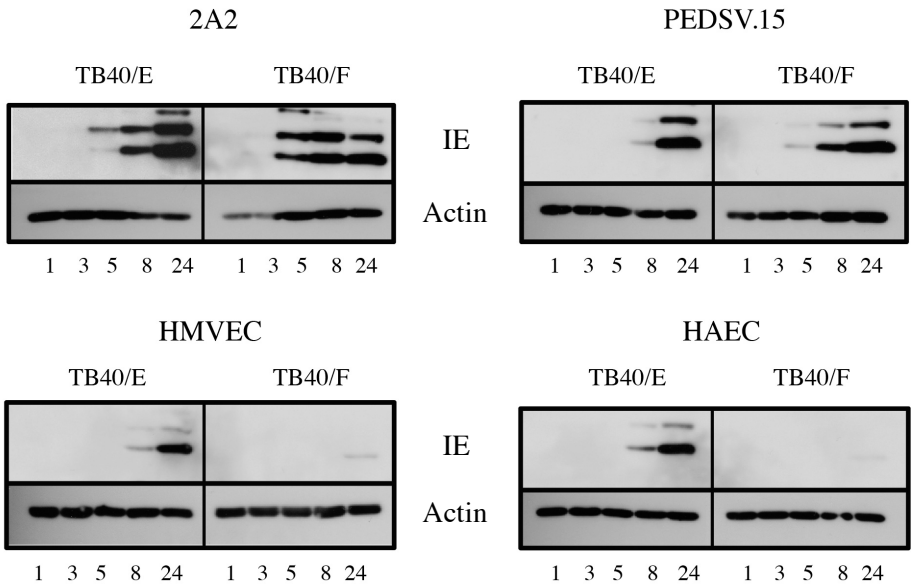

Fig. 23: Time course of infection in TB40/E- compared to TB40/F-infected EC. EC were infected with TB40/E or TB40/F at an MOI of 1 and the expression of IE viral proteins was detected by Western blot at different times post infection (1 to 24 hpi). The membranes from Fig. 20 were stripped and then stained with an anti-IE proteins antibody. β-actin was used as a loading control.

6.3.4 Involvement of Focal Adhesion Kinase in HCMV translocation into human EC

As described above, it has been documented that fibrotropic strains of HCMV do not translocate to the nucleus of hEC and therefore cannot replicate in these cells. It has been reported that the activation of Focal Adhesion Kinase (FAK) is important for entry and nuclear translocation of Kaposi's Sarcoma Associated Herpesvirus (152) and Herpes Simplex 1 virus into fibroblasts (42). FAK is expressed by most cells and is phosphorylated in response to integrin engagement and growth factor stimulation (reviewed in (112)). I and others have demonstrated that integrins are involved in HCMV entry into hEC, therefore I investigated whether FAK activation (i.e. its phosphorylated form) is involved in HCMV nuclear translocation and whether its phosphorylation pattern varies between a productive infection (TB40/E) compared to an abortive infection (TB40/F). In this results section, work was focused only on hEC since no porcine antibodies reactive against phosphorylated form of FAK are commercially available. HAEC and HMVEC were infected with either TB40/E or TB40/F at an MOI of 3 and the phosphorylation state of FAK was assessed at different times post-infection (10, 30 and 60 mpi) by Western blot. As a positive control, hEC were stimulated with growth factor. No differences were observed in the phosphorylation state of FAK between TB40/E- and TB40/F-infected hEC nor in the positive control, suggesting that the culture conditions used were not optimal for this study (Fig. 24). Several other positive controls were tested in an attempt to stimulate phosphorylation, however none of the different conditions induced a significant increase in FAK phosphorylation (data not shown). Unfortunately, the culture conditions did not allow us to demonstrate any involvement of FAK in HCMV entry into hEC so far.

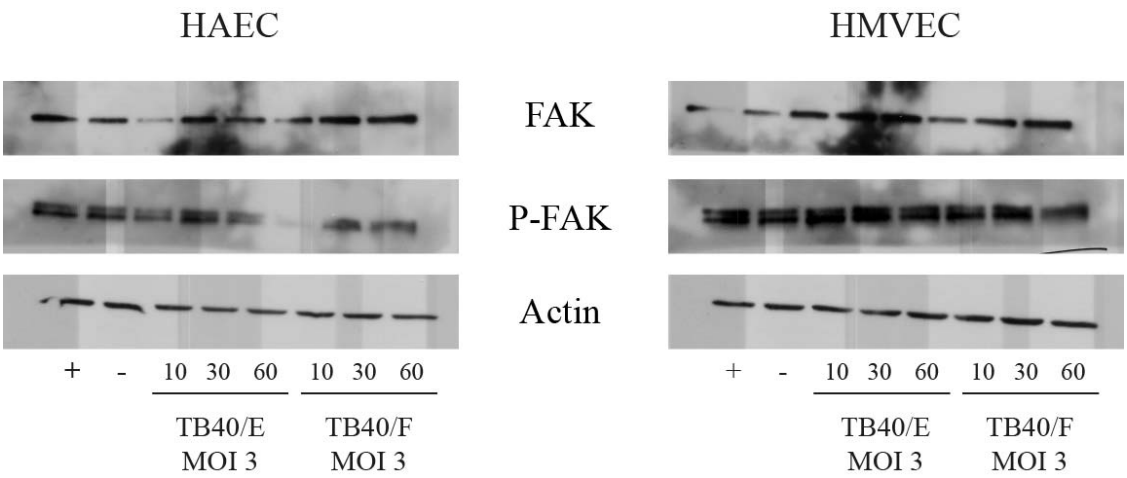

Fig. 24: Comparison of the phosphorylation pattern of FAK in human endothelial cells after infection with TB40/E or TB40/F. HAEC and HMVEC were infected with either TB40/E or TB40/F at an MOI of 3 for different times (10, 30 and 60 mpi) and the activation state of FAK (P-FAK) was assessed by Western blot. β-actin was used as a loading control. P-FAK: Phosphorylated form of FAK, +: positive control (20% FCS for 5 min). One representative experiment out of four is shown.

7 Discussion

The goal of this thesis was to address how HCMV enters cells from its own or from a different species and whether the viral strain tropism influences the entry mechanism. Degré et al. showed that pEC cells are susceptible to HCMV infection and our group expanded these findings and demonstrated that viral strain tropism and the vascular origin of pEC impact on the target cell susceptibility. This discrepancy in the efficiency of infection led us hypothesize that entry mechanisms used by HCMV depends on both the viral strain tropism and the vascular origin of target cells. For this purpose, we compared potential HCMV entry pathways of an endotheliotropic (TB/40E) and a fibrotropic (TB40/F) strain in pEC versus hEC. This work focused mainly on endothelial cells since i) they are a natural site of HCMV primary infection and ii) due to their localization (interface between the graft and the immune system), they play an important role in infectious events involved in graft rejection.

7.1 Role of known human entry cellular receptors in HCMV entry into pEC

To enter human cells, HCMV requires different receptors (PDGFRα and EGFR) (34, 272, 308), although not necessary for all settings (131, 301), and co-receptors (β1, αV and β3 integrin subunits) (81, 307). I showed that except for EGFR that is detected only in 2A2 cells, the expression of PDGFRα and the different integrin subunits is common to all four cell types used in this study. To test the involvement of PDGFRα and integrins in HCMV entry into pEC and hEC, I evaluated the consequences of silencing of these receptors / co-receptors on HCMV entry. Because of technical issues, I could only assess the involvement of β1 integrins and PDGFRα in HCMV entry into hEC and pEC, and we demonstrated that the involvement of these two cellular receptors depends on both the species origin and the viral tropism. The silencing of PDGFRα reduced the expression of IE proteins 24 hpi in pEC but not in hEC. One explanation for this discrepancy would reside in the amount of PDGFRα in the target cells. The Western blot analysis revealed that both hEC display a weaker expression of this receptor when compared to pEC, which would suggest that PDGFRα is dispensable for HCMV entry into HAEC and HMVEC as affirmed by Vanarsdall et al. (301). In this study I only used pEC from

microvascluar and aortic origin (2A2 and PEDSV.15 cells, respectively), so we cannot exclude that PDGFRα might not be involved in HCMV entry into other porcine cells as it has been observed with different human cell types.

In contrast to PDGFRα, silencing of β1 integrins resulted in a reduced expression of IE proteins 24 hpi in hEC. Similar results were observed for PEDSV.15 cells. On the contrary, β1 integrin subunit silencing had no effect on HCMV entry into 2A2 cells. One reason for this inconsistency could be that the weaker silencing of β1 integrins in 2A2 cells might not be sufficient to interfere with HCMV entry into these cells, especially as 2A2 cells are more susceptible to HCMV infection compared to all the other cells used in this study (185). Besides, regarding 2A2 cells, one cannot exclude that the presence of EGFR on this cell type might as well play a major role in HCMV entry.

In conclusion, as for hEC, entry of both HCMV strains depends on the expression of β1 integrins on pEC (except for TB40/F and 2A2 cells). In contrast, entry of both strains depends on the expression of PDGFRα on pEC whereas entry appears to be PDGFRα-independent for hEC.

7.2 Involvement of endocytic pathways, lipid rafts and endosomal pH in HCMV entry

Enveloped viruses can use two distinct mechanisms to enter their target cells: via direct fusion of the viral envelope with the plasma membrane (example: HIV) and via fusion of the viral envelope with the endosomal membrane (example: Hepatitis C virus). Depending on the cell type, herpesviruses can use these two mechanisms. HSV-1 enters epithelial cells by endocytosis (205) but enters Vero and Hep2 cells by direct fusion at the membrane (85-87, 316). The literature has shown that HCMV can as well use these two mechanisms depending on the target cell: in fibroblasts, HCMV enters by direct fusion with the plasma membrane (54) whereas in epithelial and endothelial cells HCMV uses endocytosis (19, 238). To analyse which endocytic pathway HCMV uses to enter pEC, I assessed the effect of several inhibitors specific for the different endocytic pathways on the entry of TB40/F and TB40/E. When TB40/E-infected EC were pre-treated with inhibitors of dynamin-2, clathrin mediated endocytosis or macropinocytosis, the expression of IE proteins was inhibited at 8 hpi, suggesting that entry of TB40/E into pEC is similar to entry into hEC, i.e. entry

occurs in a dynamin-2 dependent mechanism and macropinocytosis, although macropinocytosis seemed to be less involved in entry into PEDSV.15 cells as compared to 2A2 cells. In contrast, when TB40/F-infected EC were pre-treated with the same inhibitors, a reduced IE expression was observed mainly when dynamin-2 was inhibited, which suggests that entry of TB40/F into pEC occurred mainly by a dynamin-2 dependent mechanism, which has yet to be identified. These results demonstrate that TB40/E and TB40/F use distinct mechanisms to enter their target cells. Recent studies showed that herpesviruses can also use another dynamin-2 dependent entry mechanism: phagocytosis (48, 100, 295). Since inhibition of macropinocytosis has a limited effect on TB40/F entry into pEC, phagocytosis may be the more prominent entry mechanism of TB40/F. In order to verify this hypothesis, I am currently investigating the effect of an inhibitor of phagocytosis on TB40/F entry into pEC.

The next step was to assess whether lipid rafts, which are detergent insoluble plasma membrane microdomains involved in endocytic processes and signalling, play a role in HCMV entry into hEC and pEC. It has been demonstrated that several viruses use these microdomains to enter their target cells, amongst them HCMV (139, 307), HSV-1 (15) and the *Picornaviridae* member echovirus 1 (219). In contrast, the human Papillomavirus type 16 (246) and KSHV (227) do not seem to require lipid rafts to enter their target cells. For this purpose, I used a drug that disrupts lipid rafts and I analysed its effect on entry of both strains in hEC and pEC. The results showed that disruption of lipid rafts inhibited IE protein expression at 8 hpi in TB40/E - but not TB40/F-infected EC, which demonstrates that entry of TB40/E into EC depends on the presence of lipid rafts whereas entry of TB40/F into pEC does not depend on the presence of such microdomains. My finding suggests that the viral tropism might influence the dependency of lipid rafts of HCMV to enter cells of its own or another species.

Finally, it is known that viruses gain access into the host cells in a pH-independent mechanism by direct fusion at the plasma membrane or after fusion with the endocytic vesicle following exposition to low pH. A novel entry pathway involving an endocytic-dependent, pH-independent fusion has been described for HSV entry into murine melanoma cells (187), adding a new entry pathway to the endocytic pH-dependent and fusion pH-independent mechanisms (85-87, 205).

Regarding HCMV, Sinzger et al. demonstrated that TB40/E entry into human umbilical vein endothelial cells (HUVEC) occurs by endocytosis in a pH-independent manner (262), adding still another mechanism to the first two listed above. In order to determine the involvement of endosomal pH acidification in entry of both strains in hEC and pEC, I used an inhibitor of endosomal pH acidification and assessed its effect on infection. My study clearly demonstrated that TB40/E entry into hEC and pEC is pH-dependent, which was associated with an inhibition of IE protein expression 8 hpi, whereas a low pH is not a requirement for TB40/F entry into 2A2 cells and to a lesser extent entry into PEDSV.15 cells, associated with a lower inhibition of IE protein expression. These findings demonstrate that involvement of the endosomal pH acidification in HCMV entry depends as well on both the vascular bed origin of target cells and viral strains.

In conclusion, TB40/E entry mechanisms are conserved in a normal versus a cross species infection and is dependent on dynamin-2, pH and lipid rafts. In contrast, the entry mechanisms of TB40/F into pEC differ from the entry mechanism of TB40/E in the fact that these mechanisms are independent on pH and lipid rafts.

7.3 Involvement of cellular cytoskeleton in HCMV internalization and translocation

The cellular cytoskeleton is composed of three different components, namely microtubules, actin microfilaments and intermediate filaments (IF). In order to assess the involvement of microfilaments and microtubule cytoskeleton in HCMV strains entry into hEC and pEC, I used inhibitors of actin and microtubules polymerization and analysed their effect on HCMV infection. My results are in accordance with literature reporting that actin and microtubule cytoskeletons are required for HCMV entry into human cells (19) and nuclear translocation of the viral capsid (207), respectively. In pEC, I demonstrated that the actin cytoskeleton is more prominent for TB40/E entry into 2A2 cells whereas for PEDSV.15 cells, the microtubule cytoskeleton is more important. It was reported that another herpesvirus, namely KSHV, uses either actin or microtubules to infect cells, depending on the target cell (105, 202). The pEC used in this study have a different vascular bed origin, which could explain the differences observed in the involvement of the cytoskeleton in HCMV entry into these cells. Besides the origin of the cell type, the viral strain may

also influence the role of the cytoskeleton. Indeed TB40/F does not use actin cytoskeleton to enter pEC; and microtubules are not and partially involved in TB40/F infection of 2A2 and PEDSV.15 cells, respectively. Since actin microfilaments and microtubules are dispensable for TB40/F entry into pEC, another component of the cellular cytoskeleton may be involved. Currently little is known about the involvement of IF in virus entry, however, in 2009, Miller et al. reported that an intact vimentin cytoskeleton (one component of IF) is required for HCMV entry into fibroblasts (186), which would suggest that TB40/F could use the same mechanism to enter and traffic into pEC. However, inhibitors of microtubules and microfilament affect IF organization, therefore one must be cautious in the interpretation of such results (224).

In conclusion, in accordance with the literature, I demonstrated that TB40/E internalization and translocation in hEC depends on the actin and the microtubule cytoskeleton, respectively, and similar mechanisms occur for PEDSV.15 cells. In contrast for 2A2 cells, the microtubule cytoskeleton is less important. For TB40/F, contrary to TB40/E, these two cytoskeletons are dispensable for internalization and traffic into pEC.

7.4 Involvement of the host species in HCMV translocation and initiation of replication

After formation, endocytic vesicles traffic further into the cytoplasm and fuse with vesicles from the endolysosomal pathway in order to transfer their cargo (for instance virions) to more acidic compartments such as early endosomes and lysosomes. Besides guiding viral particles to their replication site, the endolysosomal pathway provides an appropriate environment to viruses for uncoating and nucleocapsid release, which is essential for a productive infection. My results showed that early after infection, internalized viral particles locates successively in $EEA1^+$ and $LAMP2^+$ compartments, suggesting that HCMV uses the classical endolysosomal pathway for intracellular trafficking. In order to better characterize intracellular trafficking events, it would be interesting to evaluate the involvement of two molecules: Rab5 and Rab7. These two small GTPase are important for vesicular traffic through the endolysosomal pathway: Rab5 is important for the delivery to early endosomes and Rab7 is essential for the maturation of late endosomes (271). Their

involvement can be evaluated with specific SiRNA or with dominant negative mutants. The study of the involvement of Rab7 would also confirm the involvement of endosomal pH acidification in entry of HCMV. Regarding the results obtained with the vacuolar H^+-ATPase inhibitor (BafA1), I would think that entry of TB40/E would be dependent on Rab7. In contrast, entry of TB40/F into pEC would be independent of Rab7, although TB40/F viral particles localize in late endosomes as determined by colocalization with LAMP2, which would confirm that late endosome maturation and consequently endosomal acidification are not required for TB40/F entry into pEC.

The tegument protein pp65 is an essential structural protein that carries two nuclear localization signals (NLS) (91, 248). At very early times after infection (i.e. 1 hpi) and before the onset of viral gene expression, pp65 originating from the entering virus is detected in the nucleus of infected human peripheral blood leukocytes (230) and human fibroblasts (11). This rapid nuclear translocation suggests an important role of this tegument protein in the initiation of viral gene expression and replication. Our laboratory has previously demonstrated that pEC susceptibility to HCMV depends on both the vascular bed origin of the cells and viral tropism. All pEC demonstrated a higher susceptibility to the fibrotropic strains compared to the endotheliotropic strains (185). In the results section, I showed that depending on the cell origin (normal versus cross—species infection) and the viral tropism, the entry mechanisms diverge. Therefore I then assessed whether nuclear translocation of both strains occurs in a similar manner in hEC and pEC. For this purpose I infected hEC and pEC with either TB40/E or TB40/F and at short times post infection, the localization of pp65 was assessed by immunofluorescence. pp65 was only detected in the nucleus of TB40/F-infected pEC. It is of note that pp65 could be detected in the nucleus of TB40/E-infected EC when high MOI were used (data not shown). I then demonstrated, by Western blot, that higher amounts of pp65 were detected in TB40/F-infected EC and PCR analysis showed that more viral DNA is detected in TB40/F-infected compared to TB40/E-infected EC. Because Taylor et al. showed a delayed expression of IE proteins in viral particles lacking pp65 (291), I assessed whether the discrepancy observed in the accumulation of pp65 in TB40/E- and TB40/F-infected EC would result in a different initiation of expression of IE proteins. My results showed that the expression of IE proteins occurs earlier in TB40/F-infected pEC.

However, translocation and accumulation of pp65 might not be linked to the initiation of IE proteins. Indeed Schmolke et al. demonstrated that pp65 is dispensable for viral growth since viral particles lacking pp65 replicates as efficiently as control viral particles expressing this tegument protein (249). Besides, Dal Monte et al. showed that a reduced expression of pp65 in cells expressing an antisense RNA to UL83 (gene encoding for pp65) inhibits viral replication. However, they observed that their antisense RNA also reduces the expression of pp71, another tegument protein. They concluded that the effect they observed was most likely due to the inhibition of pp71 expression rather than the reduced pp65 expression (62). It has been demonstrated that pp71 also localize in the nucleus of infected cells early during infection (122); therefore, in my settings, one can think that the higher accumulation of pp65 in TB40/F-infected pEC might as well correlates with higher amounts of pp71, which would be responsible for earlier IE proteins expression. One possible experiment to verify this hypothesis would be to assess the efficiency of initiation of replication of TB40/F particles lacking pp71 expression produced from bacterial artificial chromosome.

In conclusion, a marked difference was observed in the translocation of both strains in EC from human and porcine origin. The most surprising observation was the absence of nuclear accumulation of pp65 tegument protein in TB40/E-infected hEC and pEC. In contrast a nuclear accumulation was observed in TB40/F-infected pEC which correlates with higher amounts of pp65 accumulation, higher viral DNA copies and earlier initiation of replication. These observations are likely due to higher amount of pp71 whose expression is correlated to the expression of pp65 since pp71 can be transcribed as the second open reading frame of a bicistronic mRNA encoding for pp65 (235).

Finally I investigated the limiting step for nuclear translocation of TB40/F in hEC. Indeed, as already mentioned, fibrotropic strains do not replicate in hEC because of the incapacity of such viral particles to translocate in the nucleus of infected hEC (265). The literature shows that nuclear translocation of two other herpesviruses, i.e. KSHV and HSV-1 and HSV-2, is dependent on the activation of FAK. This kinase, located in focal adhesion, participates in the regulation of microtubules function and controls the actin cytoskeleton (reviewed in (245)). Therefore I assessed whether FAK

is involved in HCMV nuclear translocation and whether the activation of FAK depends on HCMV viral tropism. For this purpose I infected hEC with either TB40/E or TB40/F for different periods of time and I compared the activation profile of FAK in TB40/E- and TB40/F-infected hEC. In my settings I was not able to detect any differences neither in the activation profile of TB40/E-, TB40/F-infected hEC nor in the positive control. Therefore I was not able to investigate the involvement of FAK in HCMV entry into hEC. However, one could speculate that the absence of modulation of FAK phosphorylation might be correlated to the absence of a relationship between HCMV entry and FAK and the involvement of another kinase, maybe related to FAK, which complements some functions of FAK (259): the proline-rich tyrosine kinase-2 (Pyk2). Pyk2 and FAK share a similar domain structure and common phosphorylation sites. Besides, Pyk2 is highly expressed on EC amongst others (288) and its activation is regulated by intracellular calcium (Ca^{2+}) signal (322). Nokta et al. demonstrated that shortly after infection (1 hpi), an excessive influx of calcium occurs in human fibroblasts (206). It is possible that this Ca^{2+} influx might activate Pyk2 which would consequently facilitate HCMV entry. This alternative pathway is involved in KSHV entry into target cells in the absence of FAK (152).

7.5 Possible strategy for the prevention of HCMV infection of pEC

Because of the absence of a common entry pathway for all the cell types and the viral strains, preventing HCMV infection of pEC by way of cell manipulation will be challenging. A more suitable and more efficient approach to impair entry might be to aim at the initial entry step common to all cell types and strains, i.e. binding to HSPGs. Our laboratory has investigated the protective capacity of low molecular weight dextran sulfate (DXS) on EC. Millard et al. investigated the antiviral properties of low molecular weight DXS and they demonstrated that DXS prevents HCMV infection of pEC *in vitro* (personal communication). Besides its antiviral activity, it has been previously demonstrated that DXS inhibits the complement system (Wuillemin, 1953-1960) and the coagulation cascade (Wuillemin, 12913-12918). In the context of xenotransplantation, it was shown that DXS i) protects pEC from NK cells cytotoxicity (Laumonier, 838-843) and ii) delays HAR in a model of pig-to-human xenotransplantation by inhibiting the complement cascade (Fiorante, 24-35). Therefore, pre-treating organs with DXS prior transplantation might be one possible strategy to prevent HCMV infection of pEC.

8 Conclusion

My findings demonstrate that HCMV uses distinct entry pathways that are dependent on the viral strain, on the species origin and the vascular bed origin of the target cells. The remarkable ability of HCMV to enter cells from its own or another species and to initiate a productive infection further underlines the impressive adaptability of HCMV. As demonstrated by the large array of immune evasion mechanisms, HCMV is able to exploit different entry strategies. The dogma of a strict species-specificity of herpes viruses needs to be reconsidered. Given that pEC infection with HCMV leads to cytopathic effects and increased apoptosis, which most likely would lead to vasculopathy, graft damage and graft rejection, prevention of such an infection is paramount should experimental xenotransplantation ever reach a clinical application. A potential avenue is to render porcine grafts resistant to HCMV infection by blocking viral entry and propagation.

9 Annexes

9.1 Literature

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9.2 Abbreviations

ACXR: acute cellular xenograft rejection

AHXR: acute humoral xenograft rejection

AIDS: acquired immunodeficiency syndrome

AP: assembly protein

APC: allophycocyanin

ART: antiretroviral therapy

BafA1: bafilomycin A1

CDV: cidofovir

CMV: cytomegalovirus

CPE: cytopathogenic effect

CVB-5: Coxsackievirus B5

DAPI: 4',6-diamidino-2-phenylindole

DNA: Deoxyribonucleic acid

Dpi: day post infection

E: early (HCMV genes)

EC: endothelial cells

EEA1: early endosome antigen 1

EGFR: epidermal growth factor receptor

EKC: epidemic keratoconjunctivitis

EMCV: encephalomyocarditis virus

ER: endoplasmic reticulum

ERGIC: endoplasmic reticulum Golgi intermediate complex

FAK: focal adhesion kinase

FCS: fetal calf serum

FOS: Foscarnet

FX: factor X

gB: glycoprotein B

GCV: ganciclovir

hAdV: human adenovirus

HAEC: human aortic endothelial cells

HAR: hyper acute rejection

HBSS: Hank's balanced salt solution

HCMV: human cytomegalovirus

HCV: hepatitis C virus

hEC: human endothelial cells

HHV: human herpesvirus

HIV: human immunodeficiency virus

HMVEC: human dermal microvascular endothelial cells

Hpi: hours post infection

HSPG: heparansulfate proteoglycans

HSV-1/2: herpes simplex virus 1/2

HUVEC: human umbilical vein endothelial cells

HVEM: herpes virus entry mediator

IBMIR: instant blood-mediated inflammatory reaction

IE: immediate early

IF: intermediate filament

IFN: interferon

KSHV: Kaposi's sarcoma-associated herpesvirus

L: late (HCMV genes)

LAMP2: Lysosome-associated membrane protein 2

mC-BP: minor capsid protein-binding protein

MCMV: mouse cytomegalovirus

MCP: major capsid protein

mCP: minor capsid protein

MHC-I: major histocompatibility complex class I

min: minutes

MOI: multiplicity of infection

Mpi: minutes post infection

MTOC: microtubule-organizing center

MxA: myxovirus protein A

ND10: nuclear domain 10 (also referred to as PML)

NLS: nuclear localization signal

pAP: assembly protein precursor

PBS: phosphate buffered saline

PCMV: porcine cytomegalovirus

PCV: porcine circovirus

PCVAD: porcine circovirus associated disease

PCVD: porcine circoviral disease

PDGFRα: platelet-derived growth factor receptor alpha

PE: phycoerythrin

pEC: porcine endothelial cells

PERV: porcine endogenous retrovirus

PFA: paraformaldehyde

PKC: protein kinase C

PLHV: porcine lymphotropic herpesvirus

PML: promyelocytic leukaemia nuclear

PMWS: post weaning multisystemic wasting syndrome

PRV: pseudorabies

Pyk2: proline-rich tyrosine kinase-2

RCMV: rat CMV

RNA: ribonucleic acid

SCP: smallest capsid protein

SiRNA: small interfering RNA

SPF: specific pathogen free

T1D: type 1 diabetes

TBS: Tris-buffered saline

TGEV: transmissible gastroenteritis coronavirus

TFPI: tissue factor pathway inhibitor

UL: unique long sequence

US: unique short sequence

VCAM-1: vascular cell adhesion molecule 1

VGCV: valganciclovir

vWF: von Willebrand Factor

XNA: xenoreactive natural antibodies

αGal: α(1,3)-galactosyltransferase

9.3 Curriculum Vitae

First name(s), surname	Aline Marie TAVEIRA
Date of birth	25.02.1986
Place of birth	Tourcoing, France

Education

10/2009 – 10/2013	**University of Zurich, *Zurich, Switzerland*, PhD student** Group of Prof. Nicolas Mueller Thesis title: "Comparison of Human Cytomegalovirus Entry Mechanisms into Porcine and Human Endothelial Cells"
09/2008 - 06/2009	**Paul Sabatier University, *Toulouse, France*, Master student** Group of Dr. Daniel Dunia Master of Science in Immunology and Infectious Diseases, *2nd year* Title: "Impact of Borna disease virus on chromatin remodeling"
09/2007-06/2008	**Paul Sabatier University, *Toulouse, France*, Master student** Group of Prof. Joost van-Meerwijk Master of Science in Immunology and Infectious Diseases, *1st year* Title **:** "Allograft tolerance induction by CD4+ CD25+ T regulatory lymphocytes"
09/2004 - 06/2007	**Paul Sabatier University, *Toulouse, France*, Bachelor student** *Bachelor of Science in Cell Biology and Physiology*
2003-2004	**High school Pierre de Coubertin, *Font Romeu, France*** Scientific section with special subject physics and chemistry
2001-2003	**High school Victor Hugo, *Marseilles, France***

Awards / Presentation

2013	"Entry of Human Cytomegalovirus into endothelial cells is species-specific and depends on both cell origin and viral tropism", manuscript submitted
2011	Speaker at the "13th International CMV / Betaherpesvirus Workshop", Nuremberg, Germany Awarded as a young scientist by the FEMS (Federation of European Microbiological Societies)

Lectures

2012	Marketing and Pharmacoeconomics Discovering management

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